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PROPERTIES AND SUPERCRITICAL CARBON DIOXIDE EXTRACTION OF SEAWEED LIPIDS RICH IN OMEGA-3 FATTY ACIDS

by

Vijay Kumar Mishra

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

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submitted by **Vijay Kumar Mishra**
in partial fulfilment of the requirement for the
degree of **Doctor of Philosophy**
in **Food Engineering**

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To

My Beloved Parents

ABSTRACT

Seaweeds are good sources of ω-3 fatty acids, which have potential health benefits. Dulse (Palmaria palmata) was studied as a raw material for the production of lipids rich in ω-3 fatty acids using supercritical carbon dioxide (SC CO_2) extraction. Comparison of wild and the cultured mutant showed similar lipid content (1.9%, dry basis) and eicosapentaenoic acid (EPA, 20:5 ω-3, 45%) contents. The growth conditions that favoured maximum accumulation of lipids were 11°C and 20 days old culture which was adequately supplemented with nitrogen.

The alga was freeze dried and ground to facilitate extraction of lipids by SC CO_2. Physical properties of dried alga indicated hygroscopic nature with a BET monolayer value of 4.8 kg/100 kg.

It was technically possible to extract lipids free from pigments and polar lipophilic components by SC CO_2 within the temperature and pressure ranges of 20.78 to 62.15 MPa and 35 to 45°C, respectively. Highest recovery of lipids occurred at low pressures within the range studied. Addition of 10% ethanol to the powder resulted in increased solubility and coextraction of green pigments and polar lipids. Fatty acid composition of hourly extracts at 62.15 MPa/45°C indicated that the concentration of EPA was depleted after the 4th hour of extraction.

Data on the physical properties and phase behaviour of ω-3 fatty acid esters in SC CO_2 are needed for the design and development of the SC CO_2 extraction process. Meissner's group contribution method and a method based on gas chromatographic retention volumes were investigated for the estimation of vapour pressures of fatty acid esters (FAE) with emphasis on ω-3 fatty acids. Vapor pressures of some ω-3 and ω-6 fatty acid esters were determined at 200-230°C. The gas chromatographic method showed that volatility of fatty acid isomers increased with an increase in the ω number.

Regular solution theory was applied for modelling of binary vapor liquid equilibria of SC CO_2 and EPA and docosahexaenoic acid (DHA) ethyl esters. The relationship between the light phase solubility of both esters and solubility parameter of SC CO_2 was determined to be logarithmic at a constant temperature. The equilibrium distribution coefficients (K) of EPA and DHA ethyl esters in light and heavy phases were estimated using the theory and compared with literature data. The model predicted the increasing trend in K values as a function of pressure for the investigated isotherms and the predictions remote from critical conditions were generally more acceptable.

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NOMENCLATURE

A	constant = $H_s/\Delta H_v$, eq (5.6)
	constant, eq (6.13)
A_s	BET surface area, m^2/kg solid
A_{wat}	molar surface area of water, m^2/kmol
a	constant, eq (3.4)
a_w	water activity, dimensionless
B	constant in eqs (5.5, 5.6, 6.13)
b	constant, eq (3.4)
C	compressibility, eq (3.2), %
	constant in BET eq
D	diameter, μ
E	cohesive energy
F_c	volumetric flow rate of gas, mL/min
f	fugacity, kPa
H	heat of adsorption, kJ/kg
H_m	enthalpy of mixing, kJ/mol
H_s	enthalpy of solution, kJ/mol
H_v	enthalpy of vaporization, kJ/mol
K	Distribution coefficient, y_i/x_i
k	ratio of accomodation coefficient and frequency factor
M	molecular weight, g/gmol
M_l	molecular weight of stationary liquid phase, g/gmol
N	Avogadro's number, 6 x 10^{26} molecules/kmol
n	number of taps
n	number of mols, eq (6.4)
n_D	refractive index at sodium D line
P	parachor, $(M*\sigma^{1/4})/(\rho_L-\rho_V)$
P	system pressure, MPa
P_c	critical pressure, MPa
p^o	vapour pressure of solute, mm Hg
R	gas constant
R	correlation coefficient

R_D	molar refraction, $(M/\rho_L)*(n_D^2-1)/(n_D^2+2)$
T	temperature, K
T_b	normal boiling point, K
t_r	retention time, min
t_{ra}	retention time for unretained sample, min
V	volume, m^3
V_{gc}	molar volume evaluated by Fedor's group contribution method, cm^3/mol
V_n	volume after tapping, eq (3.4) m^3
	net retention volume of solute, eq (5.2), mL/min
V_r	retention volume, mL
V_s	specific retention volume, mL/g
W_l	weight of stationary liquid phase, g
X	equilibrium moisture content, kg water/kg dry solid
X_m	monolayer moisture content, kg water/kg dry solid
x	mass fraction in liquid phase
y	mass fraction in vapor phase
Z	compressibility

Subscripts and Supercripts

corr	corrected
b	bulk
F	fluid phase
i	ith component
L	liquid phase
o	standard state
p	particle
0	initial state
1,2	solvent and solute, respectively
∞	infinite dilution

Greek symbols

γ	fraction of volume reduction, dimensionless

γ	activity coefficient, eqs (5.4, 6.3, 6.6, 6.7, 6.9), dimensionless
ρ	density, kg/m^3
ρ_{R1}	reduced density of supercritical fluid, kg/m^3
ρ_{RL}	reduced density of liquid = 2.66
ε	porosity, dimensionless
ϕ	fugacity coefficient, dimensionless
δ	solubility parameter, $(cal/cm^3)^{1/2}$
Φ	volume fraction, eqs (6.8, 6.10)

1. GENERAL INTRODUCTION[1]

Current interest in fish oil is due to their long chain ω-3 fatty acids, which have been proposed to have potential health benefits in reducing the risk of various diseases (Weaver and Holub, 1988), including cardiovascular diseases, a leading cause of death in North America. With an increase in public awareness of the nutritional benefits of consuming fish and seafood in general and ω-3 fatty acids in particular, the market for these products is expected to grow in the future. Nutritional studies on the effect of supplementation of ω-3 fatty acids have concluded that ω-3 concentrates were ideal with respect to their effect on various physiological parameters (Kinsella, 1986). Concentrated source of these fatty acids are desirable to achieve sustainable benefits. In response to this demand pharmaceutical industries brought forth different formulations of the fatty acids. Concentrates already available in the market have been reviewed by Ackman (1988).

1.1 Chemistry and nutritional significance of ω-3 fatty acids

Polyunsaturated fatty acids (PUFA) are classified according to the position of double bond either from the methyl end or carboxyl end. In the "omega" or "n" convention,

---

[1] A version of this chapter has been submitted for publication in Food Research International (Mishra et al., 1992).

the double bonds are counted from the methyl end of the carbon chain. A majority of PUFAs belong to ω-9 (e.g. mead acid), ω-6 (e.g. arachidonic acid) and ω-3 (e.g. eicosapentaenoic acid) groups. The first double bond in ω-3 fatty acids starts at the third carbon atom from the methyl end. The major fatty acids belonging to this family are α-linolenic acid (ALA, 18:3), eicosapentaenoic acid (EPA, 20:5), docosahexaenoic acid (DHA, 22:6) and docosapentaenoic acid (DPA, 22:5). The chemical structures of these fatty acids are given in Figure 1.1. Due to the nonconjugated (methylene interrupted) cis type double bond configuration, the carbon chain is bent at the site of double bonds and the molecules show some crumpling. These fatty acids originate in unicellular phytoplanktons and seaweeds and accumulated in fish (Ackman, 1980). The precursor of long chain ω-3 fatty acids is ALA, which is subsequently chain elongated and desaturated to EPA and DHA. Both EPA and DHA occur as constituents of fish triglycerides and phospholipids.

The pioneering epidemiological work of Dyerberg and Bang (1979) on Greenland Eskimos suggested a possible link between low incidence of heart diseases and the consumption of seafoods. Since then, many studies have been published on the role of ω-3 fatty acids in human health and diseases. These fatty acids have been reported to have beneficial effects in cardio-vascular diseases, autoimmune disorders and other inflammations (Weaver and Holub, 1988). The beneficial

effects of ω-3 fatty acids are explained through altered eicosanoid metabolism in the circulatory system leading to the production of prostaglandins considerably weaker in inducing platelet aggregation than those produced via ω-6 fatty acids. The physiological effects of ω-3 fatty acids are:

1. Lowering of plasma triglycerides,
2. Increased aggregation time for platelets,
3. Decreased viscosity of blood,
4. Decreased blood pressure,
5. Reduction in atherosclerosis,
6. Reduction of inflammation (arthritis, psoriasis, asthma), and
7. Reduction in tumors.

Prevention of cancer is still debated as unsaturated fatty acids produce free carbonyl compounds which are tumorigenic. Detailed treatment of the medical aspects has been the subject of several publications (Kinsella, 1987; Lees and Karel, 1990).

1.2 Sources of ω-3 fatty acids

ω-3 fatty acids can be found in seafoods, marine oils, seed oils and leafy vegetables. Fish and seed oils are the most important sources of ω-3 fatty acids. However, non-conventional sources e.g. macro and microalgae are being identified as potential sources long chain PUFA, particularly EPA and DHA.

1.2.1 Fish oil

Total world production of fish oil in 1988 was 1.5 million metric tons (Yearbook of Fishery Statistics, 1988).Most of this oil production has been used in various food and pharmaceutical formulations. Typical food uses of hydrogenated or partially hydrogenated fish oils are production of salad oils, frying oils, table margarines, low calorie spreads and shortening in bakery products (Bimbo, 1990). Major share of US production is exported overseas. Except for hydrogenated menhaden oil, the food use of fish oil is limited due to its high PUFA content leading to poor stability; presence of biologically active antagonistic fatty acids e.g. erucic acid (22:1), which is a suspected nutritional hazard; presence of toxins e.g. biphenyls; and wide variations in the quality of oil. The lipid content of fish varies immensely with fish species, season and location of the catch, food habitat and part of body. The fat content of low to high fat containing fish ranges from 0.5 to 24% (Kinsella, 1990). The fatty acid profile of selected fish oils is given in Table 1.1. Fish oils contain saturated, monounsaturated and polyunsaturated fatty acids. The predominant fatty acids are 14:0, 16:0, 18:0, 18:1, 22:1, 20:5 and 22:6. The EPA and DHA contents of fish oil usually vary from 5 to 24% and 4.1 to 37%, respectively (Kinsella, 1986). Fish oils used for concentration of ω-3 fatty acids have been obtained from Pacific herring (Jangaard, 1966), cod liver (Haagsma et al.,

1982; Rizvi et al., 1988), menhaden (Ratnayake et al., 1985; Rizvi et al., 1988) and red fish (Ackman, 1988). Ackman et al. (1988) found that Atlantic red fish, Atlantic herring, and Pacific dogfish could be used for enrichment of ω-3 fatty acids since the concentrations of ω-3 fatty acids in all these fishes were similar.

1.2.2 Seed oils

Flax (58%), canola (9%) and soybean (8%) constitute major sources of ALA (18:3,ω-3), which can be converted to EPA and DHA in the human system. Since the conversion rate of ALA to physiologically more active EPA and DHA is extremely poor in the human (Dyerberg, 1986), flax oil is currently being used as an animal feed to increase the ω-3 fatty acid content of pond fish, pork and poultry eggs. Studies involving incorporation of flax or flax oil in food formulations are ongoing (Anon., 1990).

1.2.3 Algal lipids

Marine fish feed and accumulate ω-3 fatty acids from marine phytoplanktons and seaweeds. Therefore, the latter are the ultimate source of these fatty acids. Advantages offered by these organisms as a source of lipids are their ability to grow on an artificial culture media and amenability for genetic manipulation for the production of desired end products. With the depleting fish stocks, it may be difficult to fulfil the demand for these fatty acids by fish oil alone, therefore, micro and macroalgae (seaweeds) are being con-

sidered as an alternate non conventional source of ω-3 fatty acids (Ackman, 1981; Ackman and McLachlan, 1979; Choi, et al., 1987; Polak et al., 1989; Radmer, 1990; Kyle, 1991). Although the lipid content of algae generally ranges from 0.5 to 1.5% depending on the type, season and cultural conditions (Ackman, 1981), the lipids are rich in ω-3 fatty acids (Table 1.2). It can be noted from Table 1.2 that algal fatty acids are predominantly EPA or DHA. In some algal species, either DHA (e.g. Gonyaulax cantanella, about 34% DHA), or EPA (e.g. Palmaria palmata and Schizymenia dubyi, 71% and 50%, respectively) are preferentially accumulated. High ω-3 fatty acid content of marine algae make them a suitable alternative to fish oil as a source of these PUFA. Work is ongoing for selection, identification, and genetic engineering of algae for overproduction of oil rich in ω-3 fatty acids. Some success has already been reported in this direction (Radmer, 1990; Kyle, 1991). It has also been mentioned that oil from microalgal sources had better oxidative stability than fish oil due to the presence of some unidentified components (Radmer, 1990).

1.3 Fish oil extraction and production of ω-3 fatty acid concentrates

Fish oil has been used exclusively for isolation and concentration of ω-3 fatty acids. The first step in this operation is extraction of oil from good quality fish catch.

After mincing, fish is cooked at suitable conditions (90°C for 15 min) to denature proteins and release the oil (Kinsella, 1987). After filtration and washing, crude oil is subjected to several refining steps, i.e. degumming, neutralization, bleaching, and deodorization. Stability of oil is a major problem in a fish processing plant. Autoxidation of PUFA gives rise to highly unstable hydroperoxides which ultimately produce off flavors. The oil is stored in a sealed container with an inert atmosphere at low temperature to avoid autoxidation. Antioxidants are frequently added to further ensure stability.

1.3.1 Conventional methods of preparation of PUFA concentrates

Fish oils are complex mixtures of compounds which vary in chain length and degree of unsaturation. Separation of individual components is a difficult task and a high degree of enrichment is not feasible. Fish oil triglycerides are often hydrolyzed to constituent fatty acids and converted to either methyl or ethyl esters. The difference in chain length and degree of unsaturation of fatty acids and their esters can be easily exploited for separation and fractionation. The rest of the discussion will be centred on the methods employed for concentration of either fatty acids or their methyl or ethyl esters.

The methods that have been used for concentration of ω-3 fatty acids are crystallization, counter current distribution, fractional or molecular distillation, adsorption chromatogra-

phy and supercritical fluid extraction (SFE) and chromatography. The principles underlying these separation methods have been covered by Stout et al. (1990). Most often above methods are employed in conjunction with one another.

Schemes based on vacuum distillation (Jangard, 1966), urea complexation and vacuum distillation have been described by several researchers (Haagsma et al., 1982; Ackman, 1988; Ratnayake et al., 1988). The process of concentration essentially consists of saponification of fish oil to release fatty acids, urea complexation and separation of complexes, esterification of the uncomplexed fraction rich in ω-3 PUFA and vacuum distillation, typically at 250°C and 0.2-0.5 torr (Ratnayake, 1988).

Exposure of these PUFA to high temperatures during distillation at 250°C produces thermal degradation products of PUFA. Chemical reactions that may take place at these temperatures are hydrolysis, thermal oxidation, polymerization and isomerization. Degradation end products of these reactions are cyclic fatty acids and high molecular weight polymers. Ackman (1988) found that prolonged heating of PUFA in a packed Stedman column at 250°C resulted in a reduction of gas chromatographic peaks of all PUFAs, including ω-3 fatty acids, indicating some degradation during distillation. Wijesundera et al. (1989) reported the presence of various geometric isomers of EPA. The identified artifacts were 20:5-Δ*trans*-5, *cis*-8, *cis*-11,*cis*-14,*cis*-17; 20:5-Δ*cis*-5,*trans*-8,*cis*-11,*cis*-

14,cis-17; and 20:5-Δcis-5,cis-8,cis-11,cis-14,trans-17. Few other unidentified artifacts were also noted by the same authors. Based on these studies, it may be concluded that a method for the preparation of ω-3 fatty acid concentrates should involve low process temperature and short time to minimize thermal damage.

High performance liquid chromatography and silver resin chromatography were used for preparative separation of PUFA concentrates (Aveldano et al., 1983; Bailie et al., 1982; Halgunset et al., 1982; Rogerro and Coen, 1981; Pei et al., 1975; Adolf and Emken, 1985). Adolf and Emken (1985) enriched a commercial ω-3 fatty acid concentrate from 76.5% to 99.8% using isocratic elution from a silver resin column. The problems with chromatographic techniques are use of toxic solvents, loss of resolution on repeated use of the column and difficulty in scaling up to commercial production.

1.3.2 Supercritical fluid extraction and fractionation of fish oils and fatty acid/esters

Supercritical fluid extraction (SFE) is a relatively new unit operation which can be used to solve some of the problems associated with conventional separation techniques, e.g. distillation and solvent extraction. It is particularly relevant to food and pharmaceutical applications involving processing and handling of complex, thermolabile bioactive components. The technology has already been in commercial use

for extraction of caffeine from coffee beans and essential oils from hops (McHugh and Krukonis, 1986).

A supercritical fluid (SCF) is a fluid at a temperature above its critical point. Figure 1.2 depicts the pressure-temperature phase diagram of CO_2. The isothermal compressibility of a fluid near its critical point is infinity, which translates into rapid change in its density as a function of temperature and pressure. Hence, it is possible to vary the solvation capacity of the fluid and consequently, achieve extraction and separation of a solute within a narrow range of temperature and pressure. The region shown by dotted rectangle in Figure 1.2 is important in SFE. Under these conditions, it possesses liquid like density attributing high solvation capacities and gas like transport properties. Physical principles underlying SFE technology have been reviewed elsewhere (Irani and Funk, 1977; Williams, 1981; Paulaitis et al., 1983; Rizvi et al., 1986; McHugh, 1990). For food applications, CO_2 is the solvent of choice because it has moderate critical temperature and pressure (31°C, 7.3 MPa) and it is inert, inexpensive, readily available, and safe. Significant features of SFE technology from a viewpoint of extraction are (Wilke, 1978):

1. Involatile solutes can be dissolved.
2. Properties of SCF, e.g. density, can be varied within wide limits by a combination of temperature and pressure,

thereby achieving selective extraction and separation of solute.

3. Principles employed in distillation (vapour pressure) and solvent extraction (phase separation) are used simultaneously.

4. Gas like transport properties (high diffusivity and low viscosity) attribute high penetration power required in extraction of solubles from a solid matrix.

5. Solvent can be reused, leading to profitable economy and energy saving.

6. Possibility of extracting and fractionating bioactive components at mild temperatures.

M.W. Kellog Co. in the year 1946 announced a Solexol process for concentration of polyunsaturated triglyceride (TG) from vegetable oils and extraction of vitamin A from fish oils. Near critical propane was used as a solvent to fractionate crude oil into vitamin concentrate, fatty acids and other fractions with different iodine, saponification and acid values and colour (Passino, 1949; Dickinson and Meyers, 1952).

Supercritical fluid technology have been used in fish processing for extraction of oil rich in ω-3 fatty acids from fish and seaweeds (Yamagouchi et al., 1986; Choi et al., 1987; Hardardottir and Kinsella, 1988; Polack et al., 1989) and concentration of ω-3 fatty acids or esters from fatty acids

and esters, respectively (Eisenbach, 1984; Krukonis, 1988a, 1988b; Nilsson et al., 1988, 1989; Rizvi et al., 1988).

Physiological studies on metabolism of ω-3 fatty acids from various sources have indicated that TGs are more easily absorbed than either methyl or ethyl esters (El-Boustani et al., 1987). Triglycerides are therefore, preferred for delivery of these PUFA to human subjects. Since fish lipids are predominantly TGs, it is advantageous to extract triglycerides from their natural sources. A few studies have been reported on this subject. Yamagouchi et al. (1986) used SC CO_2 at 24.5 MPa and temperatures of 40, 50, 60°C to extract lipids from freeze-dried krill (16.7% oil) and krill meal (11.5% oil). Recovery of oil from krill meal was lower than that from the freeze dried krill due to possible denaturation and polymerization of lipids in krill meal. At constant pressure, the solubility of lipids was found to be independent of temperature. Hardarttoir and Kinsella (1988) made similar observations while investigating SC CO_2 extraction of oil and cholesterol from rainbow trout. The recovery of lipids remained constant between 13.8 to 34.5 MPa pressure and 40 and 50°C. Addition of 10% ethanol as an entrainer increased the solubility of lipids in SC CO_2. However, this resulted in coextraction of polar lipids due to a change in polarity of solvent (Hardardottir and Kinsella, 1988). The muscle proteins were reported to be denatured by high pressure extraction as well. These two studies clearly state that

interactions between the solute and the solid structural matrix are important factors in determining the solubility of lipids in SC CO_2 from fish. It is also known that lipids in fish are embedded in a lipoprotein matrix and in order to dissolve lipids, lipoprotein bonds have to be broken.

With the problems encountered in extraction of oil directly from fish, the usual approach is to extract oil from fish using conventional methods, hydrolyze the fatty acids and esterify them before subjecting to SFE. Eisenbach (1984) was the first to use SFE for concentration of C20 fraction to 95%. However, it was not possible to separate EPA from other C20 fatty acids as the maximum theoretical concentration of EPA was limited by the material balance (Krukonis, 1988a). It has been shown clearly by Eisenbach (1984) and Krukonis (1984) that fractionation of esters of fatty acids by SFE is based on carbon number. Fatty acid methyl esters have been found to be a better feedstock for concentration of ω-3 fatty acids than free fatty acids or their triglycerides, which is in accordance with the fact that low molecular weight, high volatility compounds have higher solubility at a given set of extraction conditions (Krukonis, 1984).

In a single pass system without refluxing, extraction pressure is increased in steps at constant temperature followed by separation of dissolved components by isothermal pressure reduction or by isobarically increasing temperature. By changing pressure, solubility of different components is

varied. In later studies, refluxing was added to increase the efficiency of separation (Krukonis, 1988a, 1988b; Rizvi, et al., 1988). Selectivity of one fatty acid ester over another can be varied by providing a refluxing system over the extraction vessel. Refluxing can be obtained either by varying temperature alone (Eisenbach, 1984) or by varying both pressure and temperature (Rizvi et al., 1988). Heating the solute laden SCF creates an internal reflux as the density of SCF decreases, thereby decreasing the solubility of heavy compounds, which fall back into the extraction vessel and get concentrated. Ethyl esters of C20 fractions were concentrated to a level of 90% at 15.2 MPa pressure by Eisenbach (1984) using a hot finger.

In the second approach, by changing both pressure and temperature, and pumping the condensed solute phase, a counter-current flow is created in the reflux. At extraction conditions of 9.65 MPa/35°C and reflux conditions of 9.3 MPa/95°C, concentration of ω-3 fatty acids was increased from 40.4% to 87.8% (Rizvi et al., 1988). However, concentration of EPA and DHA was only marginal. Nilsson and coworkers at the National Marine Fisheries Service, Seattle (Nilsson et al., 1988; 1989; 1991; Stout et al., 1990) have studied concentration of fish oil esters extensively. Based on the distribution coefficient (K value[2]) analysis it was confirmed

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[2] is a ratio of concentration of ester in CO_2 rich vapour phase to concentration of ester in ester rich liquid phase.

that at constant temperature and pressure of extraction, solubility decreased with the carbon chain length and remained relatively unaffected by increasing degree of unsaturation with only a slight increase at the extreme end of unsaturation. At a constant pressure (15 MPa), increasing temperature resulted in a decrease in distribution coefficient, suggesting retrograde condensation within these range of conditions. Figure 1.3 shows the effect of temperature on the distribution coefficient of various ω-3 fatty acid ethyl esters (Krukonis, 1988b). For the investigated fatty acid esters, K values decreased with temperature. The decreasing pattern was steeper at lower range of temperature (40-60°C) than at higher temperature range (80-100°C). Again, this indicates that between 40 to 60°C, retrograde condensation prevails. Also, since the difference between the distribution coefficients for ω-3 fatty acids is small at higher temperatures (80-100°C), separation of individual fatty acids differing mainly in the degree of unsaturation would be difficult.

Nilsson et al. (1988, 1989) attempted fractionation of fish oil ethyl esters by isobaric fractionation with a temperature gradient in a column and an incremental pressure programming coupled with a temperature gradient. In both approaches, internal reflux was created in a packed column by heating the column along the height from 40°C at the bottom to 100°C at the top. Fractionation of a mixture of urea pretreat-

ed fatty acid esters from menhaden oil yielded a product containing 70 and 80% EPA and DHA, respectively, at 15 MPa and a column temperature gradient of 70-100°C. However, lower temperature of the heating zone was recommended to prevent thermal degradation and to reduce the solvent to feed ratio. In the second approach, Nilsson et al. (1989) employed an incremental pressure programming with a column temperature gradient of 50°C at the bottom to 80°C at the top for fractionation. By changing the pressure from 13.1 to 17.2 MPa in preset increments, they recovered 76% EPA of the feed.

In a subsequent study, Nilsson et al. (1991) investigated the effect of addition of 5% ethanol as an entrainer on the distribution coefficients (K) and selectivity of menhaden oil fatty acid esters. Addition of ethanol increased the K values for all FAEs irrespective of chain length or degree of unsaturation. The extent of solubility increase by addition of an entrainer was quantitated by defining an enhancement factor, which was a ratio of K values for FAEs in ethanol-CO_2 and lean CO_2. The factor varied between 1.5 for C14 and C16 FAEs to 3.1 for C22 esters. Hence, the magnitude of solubility enhancement was higher for higher carbon FAEs than that for lower FAEs. It was also noticed that isomers with higher degree of unsaturation and ω number showed slightly higher K values, which may be due to higher vapour pressures of the latter. The concept of selectivity was introduced to study the effect of using ethanol as an entrainer on the fractionation

behaviour of FAE. In all cases, selectivity decreased by the addition of ethanol.

Krukonis (1988a, 1988b) described a continuous countercurrent process for fractionation of FAEs. In this scheme, a stage concept was used. Based on his computations, a total of 13 stages were required to obtain EPA of 90% purity in the overhead (extract phase) product and DHA in the raffinate. The solvent to feed ratio could be reduced to 30 (Stout et al., 1990) in comparison to 80-530 in a batch process (Nilsson et al., 1988; 1989). The cost of producing 90% EPA concentrate by this method was estimated at $108/lb, which is seemingly comparable with the costs involved in the production of concentrates using conventional methods (Stout et al., 1990). This estimate was inclusive of both capital and operational costs for a feedstock consisting of FAEs after pretreatment with urea complexation.

1.3.3 Supercritical fluid chromatography in enrichment of ω-3 polyunsaturated fatty acids/esters

Supercritical fluid chromatography (SFC) is another application of SCF technology for preparative and analytical separation and enrichment of ω-3 PUFA (Berger et al., 1988; Higashidate et al., 1990). The advantages offered by SFC are common to those described for SFE, except that separation efficiency is improved further by the introduction of another auxiliary phase. Higher separation efficiency, better control of elution of involatile compounds by pressure and temperature

programming are some of the distinct advantages of SFC over other chromatographic techniques.

Berger et al. (1988) used a 6 cm x 60 cm reverse phase (C18) packed column to enrich DHA in a feed consisting of 14.8% EPA and 73.0% DHA methyl ester. Supercritical CO_2 was used as a carrier fluid (13.5 MPa/50°C). At an injection rate of 17.5 g/h of feed three fractions were collected one after another at an interval of 20 min. While the first fraction was richer in EPA (54.6%) than DHA (26.7%), the second fraction consisted mainly of DHA (89.8%). The authors concluded that enrichment of DHA depended strongly on the feed concentration. To achieve a 90% pure DHA, a feed should be preconcentrated to at least 65% before attempting the SFC.

Higashidate et al. (1990) employed a silica gel column (12.5 cm x 1 cm) coated with silver nitrate. Prior to SFC, SFE of methyl ester of sardine fatty acids was carried out at 8 MPa/40°C and a flow rate of 9 g/min (based on liquid CO_2). A pressure programming sequence for SFC was 8,12 and 20 MPa with ethanol as an entrainer. Five fractions were collected sequentially, fraction I (8 MPa, 110 min), fraction II (8 MPa, 110-180 min), fraction III (12 MPa, 180-250 min), fraction IV (20 MPa, 250-310 min), and fraction V (20 MPa, 310-370 min). Flow rate of mobile phase was changed to 5 g/min from 9 g/min and ethanol was added (0.1 mL/min) only in the last fraction. The EPA and DHA contents of each fraction were as follows, 0% EPA and DHA in I, 57% EPA and 0% DHA in II, 93% EPA and 0% DHA

in III, 46% EPA and 18% DHA in IV, and 0% EPA and 82% DHA in V. The order of elution was in accordance with interaction of C=C with silver ion in the stationary phase. Due to these interactions fatty acids with higher degree of unsaturation will elute after those with low unsaturation.

1.4 Extraction of algal lipids

Solvent extraction is a common method of extraction of lipids from algae. The solvents in use are acetone, chloroform, methanol, ethanol, isopropanol, n-hexane, etc. (Ackman, 1981). These solvents are used as mixtures in various proportions to ensure complete extraction.

Use of SCF for extraction of algal lipids is quite recent. Choi et al. (1987) were the first to extract lipids from freeze dried green algae (Scenedesmus obliquus) protein in two steps. Supercritical CO_2 (37 MPa/40°C) was used to extract some lipids and then the residue was reextracted with SC CO_2 with 15% ethanol as an entrainer. The single step extraction yielded mostly neutral lipids with some glycolipids. As expected, use of ethanol in SC CO_2 increased the solubility of polar lipids. Polak et al. (1989) investigated SC CO_2 extraction of lipids from Skeletonema costatus (5-10% oil) and Ochronomas danica (30% oil). The EPA content of the former was higher than the latter. The extraction was carried out at 17-31 MPa and 40°C. The solubility of lipids from freeze dried algae was found to reach a maximum at 24 MPa and

the solubility did not increase by raising pressure to 31 MPa. Under the conditions of extraction, chlorophyll was not dissolved.

In conclusion, the interest in the health aspects of PUFA is growing. Omega-3 fatty acids, in particular have been identified to have a role in ameliorating various human diseases. It appears certain that demand for these bioactive compounds will grow further. With the depleting fish stocks, sources other than fish must be exploited to satisfy the market for these health products. Algae offer a variety of fatty acids including ω-3 fatty acids (EPA and DHA) in concentrations comparable to or exceeding fish oils. With new culture techniques and genetic engineering of potential producers, it may be possible in the near future to obtain algal strains with higher lipid as well as ω-3 fatty acid contents. Suitable extraction and fractionation techniques should, therefore, be developed in order to keep pace with the progress in biotechnolgy. The scientific data in this direction are urgently needed.

1.5 Objectives and Organization of the study

The present study was undertaken to study the extraction of oil rich in ω-3 fatty acids using supercritical CO_2. Dulse (Palmaria palmata) was selected as a source of these fatty acids due to its high EPA content (71%, cf. Table 1.2) and low concentration of other interfering fatty acids (no DHA). As

described earlier, initial concentration of EPA in the feedstock is extremely important for its enrichment and concentration.

The main objective of the study described in this thesis was to investigate the extraction behaviour of oil rich in ω-3 fatty acids in supercritical CO_2. The study is organized into 5 main parts:

1. Composition of P. palmata and effect of culture conditions on the lipids (Chapter 2).
2. Physical characterization of freeze dried alga, with an emphasis on physical properties relevant to storage, handling and extraction (Chapter 3).
3. Extraction of oil from P. palmata using SC CO_2, effect of temperature and pressure on the extraction of lipids (Chapter 4).
4. Vapour pressure of fatty acid esters: Correlation and prediction (Chapter 5).
5. Modelling binary phase behaviour of SC CO_2 and ethyl esters of EPA and DHA (Chapter 6).

Table 1.1. Principal fatty acids of selected fish oils (wt%).

Fatty acid	Menhaden[1]	Rainbow trout liver[1]	Atlantic herring[2]	Cod liver[1]
14:0	8.0	8.7	6.8	1.1
16:0	24.2	18.2	14.8	18.5
16:1	10.5	13.6	7.8	3.7
18:0	3.0	5.5	1.1	5.3
18:1	23.4	13.9	16.6	14.7
18:2 ω6	2.1	3.4	0.8	1.7
20:1	1.8	1.8	16.0	9.8
20:4 ω6	2.1	1.2	0.2	0.4
20:5 ω3	14.0	15.5	2.8	6.4
22:0	2.0	1.6	-	4.2
22:1	1.6	3.4	23.1	2.3
22:6 ω3	11.6	7.0	2.7	27.4
Total saturated	37.2	34.0	22.7	29.1
Total monounsaturated	37.3	32.7	63.5	29.7
Total ω6 (LA+AA)	4.2	4.6	1.0	2.1
Total ω3 (EPA+DHA)	25.6	22.5	5.5	33.8

[1] Adapted from Kinsella (1990)

[2] Adapted from Ackman (1980)

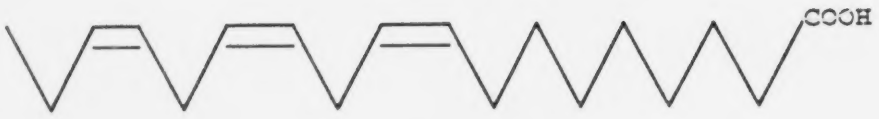
AA Arachidonic acid

LA Linoleic acid

Table 1.2. ω-3 fatty acid content of some algae.

Species	Fatty acid (wt%)				Reference
	ALA (18:3)	EPA (20:5)	DPA (22:5)	DHA (22:6)	
Amphidinium carterri	0.1	7.4	0.6	25.4	1
Dunalienlla primolecta	10.4	9.7	3.9	-	2
Cryptomonas sp.	7.0	16.0	-	10.0	3
Schizymenia dubyi	2.6	49.8	-	-	4
Chondrus crispus	5.8	26.4	-	-	4
Palmaria palmata	-	71.0	-	-	5
Skeletonema costatum	0.3	13.8	-	1.7	1
Gonyaulax cantanella	1.8	11.2	-	33.9	6

1 Ackman et al. (1968)
2 Chuecas and Riley (1969)
3 Beach et al. (1970)
4 Jamieson and Reid (1972)
5 Ackman (1981)
6 Joseph (1975)



Linolenic Acid

Eicosapentaenoic acid (EPA)

Docosapentaenoic acid (DPA)

Docosahexaenoic acid (DHA)

Figure. 1.1 Family of ω-3 fatty acids.

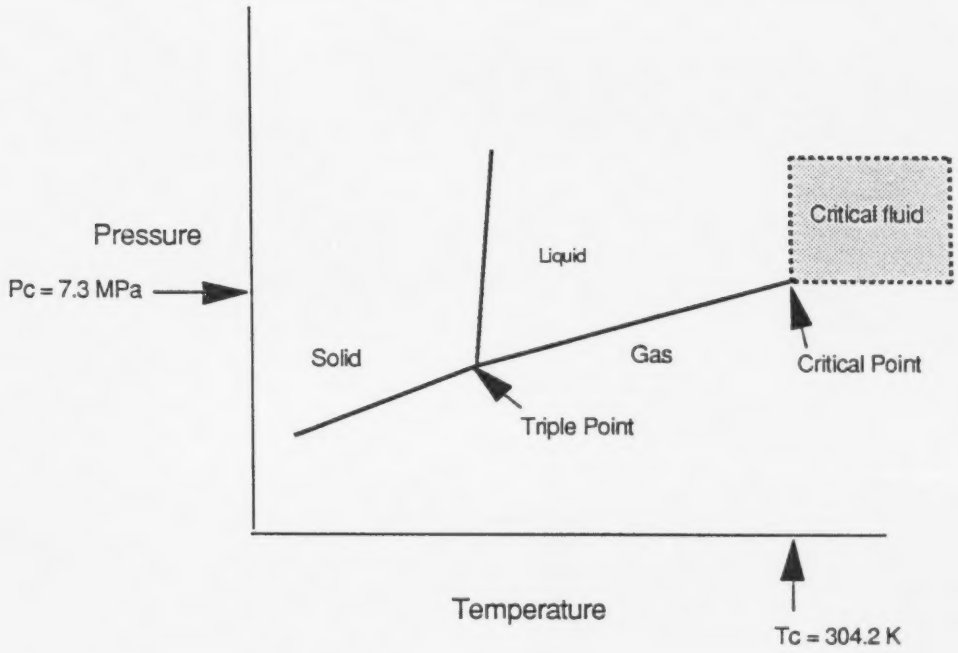


Figure. 1.2 Pressure-temperature diagram of CO_2.

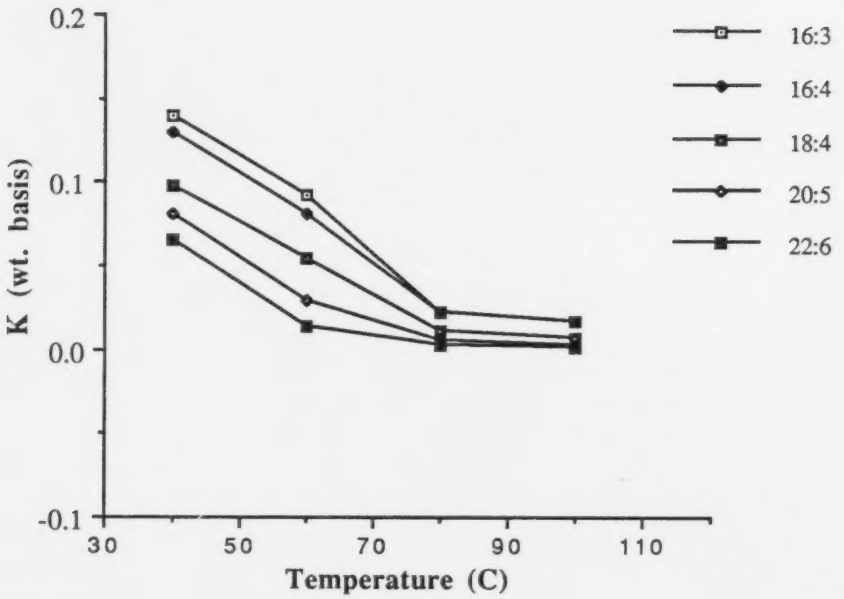


Figure 1.3. Effect of temperature on the distribution coefficients of ethyl esters of ω-3 fatty acids in SC CO_2 at 15 MPa (data from Krukonis, 1988b)

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2. LIPIDS OF THE RED ALGA, (PALMARIA PALMATA)[1]

Introduction

Seaweeds have been known as a source of food, fodder, fertilizer and pharmaceutical since ancient times (Abbott, 1988; Indergaard and Minsaas, 1991). Traditionally, consumed as food in Asian countries, they contribute a variety of nutrients to the diet (Ito and Hori, 1989) including omega-3 (ω-3) fatty acids such as eicosapentaenoic acid (EPA, 20:5,ω-3) docosahexaenoic acid (DHA, 22:6 ω-3) and α-linolenic acid (18:3, ω-3) (Wood, 1988). The ω-3 fatty acids are considered beneficial in reducing the risks associated with heart diseases such as thrombosis and atherosclerosis (Kinsella, 1986; Weaver and Holub, 1988). These fatty acids are passed up the food chain to marine fish, which accumulate them in their body fat (Ackman, 1982).

Palmaria palmata is commonly known as dulse or sol. It grows on the larger marine plants such as Laminaria spp. or on rocks in the littoral and sub-littoral zones of the north Atlantic seacoasts. This seaweed is consumed as food by the people living in Atlantic provinces. In common with other members of the red algae, Rhodophyta, dulse has been reported to contain 45-71% EPA (Ackman, 1981; Ackman and McLachlan, 1979; Wood, 1988). The EPA content of dulse not only compares

[1] A version of this study has been submitted for publication in Botanica Marina (Mishra et al., 1992).

favourably with fish oil (5-24% EPA)(Kinsella, 1986), but it is also devoid of DHA. In the light of current research activity to further understand the physiological role of ω-3 fatty acids, dulse lipids may serve as an interesting alternative source of EPA for the production of an EPA concentrate of high purity.

In order to provide a sustained supply of dulse, a technique for artificial cultivation was developed at the Aquaculture Research Station, Institute for Marine Biosciences, National Research Council, Halifax, N.S. A method to obtain a maximum dry matter yield and the effects of various environmental factors on growth were described by Morgan et al. (1980a).

In the present study, two strains of P. palmata i.e. wild and a cultured dwarf mutant, were investigated for their chemical composition with an emphasis on the lipids. The proximate composition, lipid fractions, and the fatty acid composition of the lipid fractions of the dulse samples are reported. The effects of growth conditions i.e. temperature, harvest time and nitrogen deprivation on the lipid content and the fatty acid composition were also investigated.

Materials and methods

Material

The wild strain of P. palmata was harvested from the Atlantic seacoast of Nova Scotia in July-August, when the

concentration of hydrophobic constituents reach a maximum (Ackman, 1981). A batch of 10 kg of fresh alga was received in Edmonton and freeze dried.

The cultured dwarf mutant strain of P. palmata was grown under laboratory conditions at the Aquaculture Research Station, Institute for Marine Biosciences, National Research Council (NRC), Halifax. A total of 100 kg of freshly harvested cultured strain was received in Edmonton. Both wild and cultured strain were harvested in Halifax, packed in dry ice and air shipped to Edmonton the same day. The cultured strain was exclusively used throughout this dissertation work after freeze drying.

Freeze drying of P. palmata and storage

The wild (10 kg) and cultured (100 kg) samples were washed with water and immediately frozen (-50°C) after receiving air shipment in Edmonton and dried in a RePP freeze dryer (Virtis Co. Inc., Gardiner, N.Y.) for 40 h, with platen and condenser temperatures of 20 and -50°C, respectively, at 200 mTorr. The freeze dried samples were vacuum packaged and stored below 5°C.

Analytical samples used for determination of the effect of time of harvest, nitrogen deficiency and growth temperature were blotted, weighed, and immediately frozen, lyophilized, and stored under nitrogen at -15°C at NRC, Halifax until shipment to Edmonton for lipid analysis.

Cultivation of dwarf mutant

The cultured strain was grown in 1.46 m^2 tanks in 900 L of natural seawater, at National Research Council, Halifax. The system was fertilized twice a week with ammonium nitrate and diammonium phosphate to give 1 mM N and a N:P molar ratio of 15:1. The tanks were flushed with fresh seawater at the rate of 3 tank volumes per day, in order to supply carbon and minor elements and to dampen the pH changes. The pH extremes noted in a day cycle were 7.8-9.4. The natural solar irradiation was that received from April 19 to July 4 in Halifax, N.S. The growth temperature was maintained between 10 and 14°C. The details of the culture conditions were reported by Morgan et al. (1980a).

In order to study the effect of growth conditions on total lipids and the fatty acid profile, the dwarf mutant P. palmata was grown at three temperatures, 7, 11 and 15°C in a separate experiment at Halifax. Four 40 L polyethylene tanks were filled with seawater and 800 g fresh weight of dwarf mutant P. palmata were added to each. The alga had been preconditioned for two weeks at 10-12°C. Fertilizers were added as above except that three treatments were applied per week. Growth measurements were made at 5-day intervals and samples were harvested for chemical analysis at time 0, day-10 and day-20. The biomass was readjusted to the initial 800 g at the day-10 sampling period. The samples so obtained were freeze dried and sent to Edmonton for lipid analysis.

Proximate Analysis

The moisture and ash contents were determined by vacuum drying and dry ashing methods, respectively, according to Osborne and Voogt (1978). Total nitrogen in the dried samples was determined using the micro Kjeldahl method (AOAC, 1980), and converted to protein by multiplying by 6.25. Carbohydrates and other organic constituents were obtained by subtracting protein, lipid and ash from the total weight of dry matter. All analysis were performed in triplicate for both wild and cultured strains. Student's t-test was employed for comparison of proximate analysis data at a level of significance of $p<0.05$.

Lipid Extraction and Fractionation

Lipids were extracted from freeze dried samples using the method of Bligh and Dyer (1959) with a slight modification. The freeze dried sample (8 g) was homogenized with 100 mL methanol, 50 mL chloroform and 42 mL distilled water for 15 min in Sorvall Omnimixer (Ivan Sorvall Inc., Newton, CT). To this mixture, 50 mL of chloroform, and 50 mL water were added and blending was continued for 15 min after each addition. The homogenate was vacuum filtered. After the two phases separate, the upper layer was discarded and the volume of the lower layer containing lipids was recorded. The total lipid content was obtained by evaporating the chloroform rich layer to dryness under nitrogen.

Lipids (1.5-2.0 g) were fractionated into hydrocarbons, non polar and polar lipids on a silicic acid column (250-400 mesh, Fisher Co., Fairlawn, NJ). Lipid fractions were eluted by 200 mL hexane, 150 mL chloroform and 150 mL methanol, respectively (Yamaguchi et al., 1987). Fractions were evaporated to dryness and the lipid content was determined gravimetrically. Lipid extraction and fractionation was repeated three times for both strains.

Lipids were separated into different classes by thin layer chromatography (TLC) using precoated silica gel G plates (250 μ thickness, Uniplate, Analtech, Newark, DE). The plates were developed in a solvent system consisting of hexane, diethyl ether, and formic acid (80:20:2, v/v/v) (Christie, 1984). Individual lipid bands were visualized under UV light after spraying with 0.1% aqueous solution of hemimagnesium salt of 8-anilino-1-naphthalene sulfonic acid (Sigma Co., St. Louis, MO) and identified by comparison with lipid standards. The TLC procedure was repeated twice.

Fatty Acid Analysis

The lipids in fresh seaweeds (50 g, wet basis) were saponified by refluxing with 0.5 N methanolic sodium hydroxide (250 mL) for 2 h (Ackman, 1981). The saponifiables were acidified to pH between 2 and 3 and free fatty acids were esterified to methyl esters by heating (90°C for 45 min) with 12% BF_3 in methanol (Supelco Co., Bellfonte, PA). Fatty acid methyl esters (FAME) were extracted in hexane and stored below

-40°C until further analysis by a gas chromatograph (GC) (Perkin-Elmer Model 8320, Norwalk, CT), equipped with a flame ionization detector and an integrator. A fused silica polar capillary column (30 m x 0.3 mm Stabilwax, Bellfonte, PA) was used for this analysis. The GC conditions were: carrier gas, helium; inlet pressure, 64.94 kPa; split ratio, 100:1; injector temperature, 250°C; detector temperature, 270°C; temperature program from 160 to 200°C at 3°C/min held at 200°C for 5 min and from 200 to 240°C at 2°C/min. The FAME were identified by comparison with authentic standards (Supelco Co.). Methyl ester of nonadecanoic acid (19:0) was used as an internal standard to quantitate fatty acids. Lipid extraction from fresh seaweeds was done in duplicate and triplicate GC injections of each sample were made.

In order to prevent any degradation of highly unsaturated fatty acids throughout the above analysis, the following precautions were taken. The lipid samples were stored in screw capped amber colored bottles in chloroform-methanol containing 0.2% BHT. Before closing the bottles, the headspace was flushed with nitogen to drive off any air. The samples were stored at -75°C.

Results and Discussion

Proximate chemical composition for wild and cultured strains of P. palmata are shown in Table 2.1. The marked difference observed in proximate composition between wild and

cultured dulse may reflect different culture conditions and/or genetic diversity between the strains. The mean moisture content of the samples was 84%. Protein and ash contents of the cultured dulse were significantly greater than those of the wild strain ($p<0.05$). High protein (27.7%) of the cultured strain may have resulted from the use of a high nitrogen culture medium (Benz-Amotz et al., 1985). On the other hand, the wild strain was relatively rich in carbohydrates (66.4%) reflecting the low ambient nitrogen levels prevalent in mid-summer seawater. The reported values for carbohydrates of the wild strain range from 38-74% (Morgan et al., 1980b). The average lipid content of the two strains was 1.9% (on dry basis), which is in general agreement with earlier reports (Morgan et al., 1980b). The two strains of P. palmata analyzed in this study can not be regarded as rich sources of lipids from the viewpoint of lipid content only. Similar conclusions have been reached by Ackman (1981).

The fractionation of total lipids on silicic acid column into hydrocarbon (HC), neutral (NL) and polar lipids (PL) revealed differences in HC and PL contents between the strains, though, the concentration of neutral lipids remained the same (Table 2.1). The wild strain contained significantly higher concentration of polar lipids, while the cultured strain had higher percentages of hydrocarbons and neutral lipids.

Effect of culture conditions on growth, lipid and fatty acid profile

The effect of growth conditions i.e. temperature and nitrogen deficiency on the change of biomass for P. palmata is shown in Table 2.2. Variation in biomass produced during the intervals (Table 2.2) reflect changes in the incident solar irradiances, except the -N culture which clearly showed the effects of nitrogen deprivation. The cultures at 15°C also grew relatively slowly as the temperature was supraoptimal for growth.

Fatty acid profiles of P. palmata at 7, 11 and 15°C and at harvest times of 0 (control), 10 and 20 days are summarized in Table 2.3. The nitrogen supply was in excess of that required for maximum growth. The predominant fatty acids under these conditions were myristic (14:0), palmitic (16:0) and eicosapentaenoic acids (20:5, ω-3) which constituted 64-76% of the total fatty acids. The EPA content varied from 32.6% (7°C, 20 days) to 45.8% (11°C, 20 days). This finding compares well with the 45.5% EPA reported by Ackman and McLachlan (1979) for wild P. palmata. However, in a subsequent study Ackman (1981) reported the concentration of myristic acid, palmitic acid and EPA to be 6.73, 15.24 and 71.11%, respectively. Although EPA levels may respond to environmental conditions (cf. 7°C, Table 2.3) changes may also reflect a different genetic potential of the strains. Neither cultured nor wild P. palmata synthesized docosahexaenoic acid, which is

a characteristic fatty acid of fish oils (Kinsella, 1986). For the 10 day old culture, EPA content decreased with an increase in temperature, while 11°C resulted in the maximum EPA content for the 20 day old culture. An increase in temperature resulted in a moderate increase in both saturated and unsaturated fatty acids for the 10 days old culture. This observation has been made by several researchers for other species (Holton et al., 1964; Kleinschmidt and McMahon, 1970; Patterson, 1970; Athern et al., 1983; Seto et al., 1984; and Yonmanitchai and Ward, 1991). At 7°C, EPA showed a decrease as the culture got older. However, at higher temperatures most fatty acids showed an increase from 10 to 20 days except for the C18 fatty acids.

The deficiency of nitrogen has been reported to induce higher lipid content and unsaturated fatty acids in unicellular algae e.g. Botryococcus braunii, Dunaliella bardawil, and Dunaliella salina at the expense of carbohydrate and protein synthesis (Chuecas and Riley, 1969; Benz-Amotz et al., 1985). The results of the effect of nitrogen deprivation at 11°C on the fatty acids for 10 and 20 days old cultures are presented in Table 2.4. Nitrogen deficiency certainly resulted in poor biomass production and loss of pigmentation. Although, the EPA content showed an increase with time, the increase was lower than that in the nitrogen-supplemented cultures of respective age. Comparison of respective values between Tables 2.3 and 2.4 show that saturated fatty acids (14:0, 16:0 and 18:0)

increased slightly whereas 16:1 ω-7 content increased drastically as a result of the deficiency of nitrogen in the culture media. Yonmanitchai and Ward (1991) showed that addition of nitrogen resulted in higher production of lipids as well as EPA in Phaeodactylum tricornutum.

The effect of nitrogen on the total lipid content and production of fatty acids is presented in Table 2.5. Nitrogen deprivation resulted in a lower lipid content for both harvest times at 11°C compared to the control. The production of EPA, myristic and palmitic acids in the nitrogen supplied 20-day old culture was greater than that of the nitrogen deprived culture after going through a minimum at 10 days. In general, nitrogen supplementation is required not only for the optimum growth of the alga but also for increased production of fatty acids.

Fatty acid profile of the lipid classes

The algal lipids contain both non-polar and polar lipids. The presence of triglyceride (TG), free fatty acid (FFA), polar lipid (PL), sterol, and hydrocarbon (HC) fractions was confirmed by TLC. Triglycerides are the main constituents of the lipids of macroalgae, including dulse (Williams, 1973; Ackman, 1981; Borowitzka, 1988). In addition, other polar lipids, such as phospholipids, glycolipids and sulfolipids are known to occur in seaweeds as membrane lipids. The results of fatty acid analysis from different lipid fractions are presented in Table 2.6. Myristic, palmitic,

oleic acid (18:1 ω7 and 9) and EPA were the major constituents of all three classes in both wild and cultured strains of P. palmata. However, the percentage of saturated fatty acids (14:0, 16:0 and 18:0) was greater (58-61%) in the polar fraction than that in the TG (28-30%) in both strains. Furthermore, the TG fraction contained more palmitic than myristic acid with an equal amount of stearic and myristic acid. Avarage EPA content of the TG fraction (50.7%) was higher than that of the polar fraction (26.5%). Monounsaturated fatty acids (16:1, 18:1) varied from 8.5 in PL fraction of wild strain to 18.5% in TG fraction of cultured strain. The presence of relatively high concentrations of free fatty acids in the lipid extracts may indicate some lipolytic activity during storage and/or processing of samples. The free fatty acid fraction of the cultured strain contained more EPA (64%) and had lower saturated fatty acid content (22.2%) than the wild strain. Polyunsaturated fatty acids of P. palmata, including EPA, are distributed in TG, polar lipid and free fatty acid fractions.

Conclusions

There was no difference in the lipid contents of wild and cultured strains of P. palmata. However, cultured P. palmata contained higher hydrocarbon and non-polar lipid fractions in comparison to the wild strain.

The total lipid and EPA contents were maximum for the 20 days old culture grown at 11°C with nitrogen supplementation. Even though it was possible to maximize the lipid and EPA contents by optimizing the growth conditions, the total lipid content remained low. For P. palmata to be a viable source of EPA, further work is needed to increase the lipid content possibly through genetic engineering.

Table 2.1. Proximate composition of *P. palmata* [a]

Component		Wild	Cultured[c]
Moisture (wt%)		83.8	84.3
Lipid (%db)		2.0	1.8
Fractions (%lipid)	Hydrocarbons*	2.8	4.8
	Nonpolar	13.8	16.4
	Polar*	80.3	70.3
Protein (%db)*		16.8	27.7
Ash (%db)*		14.8	27.1
Carbohydrates and others[b]*	(%db)	66.4	43.4

* indicated significant differences at p=0.05 level.
a data are mean of triplicates.
b obtained by difference.
c culture conditions 10-14°C, nitrogen suplementation, harvested on May 22, 1990.

Table 2.2. Effect of temperature and nitrogen deprivation on the growth of P. palmata at a 5 day interval as percent increase in biomass per 5 day[1].

Temperature (°C)	After 5 days	After 10 days	After 15 days	After 20 days
7	28.6	27.9	41.5	29.4
11	32.5	24.4	43.2	33.3
11 (-N)	20.8	10.3	8.3	6.4
15	23.9	14.2	26.9	13.0

[1] Data are provided by Dr. J.S. Craigie, National Research Council of Canada, Halifax, N.S.

-N indicates nitrogen deprivation.

Table 2.3. Effect of temperature and harvest time on fatty acid profile (% wt) in *P. palmata* in culture.

Fatty acids	0 day	10 days			20 days		
		7°C	11°C	15°C	7°C	11°C	15°C
14:0	6.4	6.1	6.2	5.7	8.8	7.2	7.1
16:0	22.7	20.5	22.6	22.8	25.8	23.0	24.2
16:1ω7	5.2	5.1	5.2	7.0	9.2	4.3	7.6
16:1ω5	1.2	1.6	1.3	1.4	1.5	1.4	0.9
18:0	1.1	1.3	1.3	2.1	1.5	1.7	1.8
18:1ω9	2.8	3.1	2.8	3.8	3.0	2.6	2.5
18:1ω7	5.2	7.1	7.9	9.7	8.2	3.8	8.8
18:2ω6	1.4	2.0	2.0	1.5	2.4	1.3	1.5
NI	1.1	1.1	1.3	1.3	1.2	1.4	1.1
18:3ω6	1.1	1.3	1.3	1.4	0.0	TR	1.0
18:4ω3	2.2	3.3	3.7	3.8	3.0	1.7	2.5
20:4ω6	2.6	2.6	3.5	3.5	2.6	4.4	4.8
20:5ω3	46.9	44.8	40.0	35.6	32.6	45.8	44.0

NI not identified.

TR indicates trace

Table 2.4. Effect of nitrogen deprivation and time of harvest on fatty acid profile of P. palmaria at 11°C.

Fatty acid	10 days	20 days
14:0	6.6	7.3
16:0	23.7	23.4
16:1 ω-7	10.1	7.3
16:1 ω-5	1.5	1.2
18:0	2.0	2.1
18:1 ω-9	3.4	4.5
18:1 ω-7	8.2	5.6
18:2 ω-6	1.7	1.2
NI	1.5	1.7
18:3 ω-6	1.7	1.3
18:4 ω-3	3.4	2.0
20:4 ω-6	2.3	2.4
20:5 ω-3	34.0	39.8

Table 2.5. Effect of nitrogen supplementation and time of harvest on the production of lipid (%db) and fatty acids (mg/100g dry matter) in P. palmata at 11°C.

Lipid/fatty acid	0 day	10 days		20 days	
		+N	-N	+N	-N
Lipid	0.97	0.56	0.53	0.57	0.37
14:0	21.1	15.7	27.5	36.2	24.9
16:0	71.2	55.3	99.2	111.8	78.6
16:1 ω-7+5	21.1	15.7	46.0	27.9	30.6
18:1 ω-9+7	27.4	26.4	47.5	37.3	34.5
20:5 ω-3	157.0	98.3	141.7	205.0	136.3
Others	34.4	33.9	49.5	48.7	42.1

+N nitrogen supplementation

-N nitrogen deprivation

Table 2.6. Major fatty acid composition (wt%) of lipid classes of P. palmata.

Fatty acid	Polar lipid		Triglyceride		Free fatty acid	
	Wild	Culture*	Wild	Culture*	Wild	Culture*
14:0	14.6	11.0	4.8	4.6	8.0	4.0
16:0	44.7	45.4	18.8	20.7	39.8	16.5
16:1 ω-7+5	1.5	7.6	1.9	1.6	3.7	2.8
18:0	1.6	1.6	4.7	5.2	4.5	1.7
18:1 ω-9+7	7.0	9.2	14.1	16.9	10.4	7.0
18:2 ω-6	1.5	1.4	2.3	-	1.8	2.1
20:4 ω-6	-	-	3.1	TR	2.0	2.0
20:5 ω-3	29.1	23.9	50.3	51.1	27.3	63.9

* growth conditions 10-14°C, nitrogen supplementation, period of harvested on May 22, 1990.

TR indicates trace

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3. PHYSICAL CHARACTERIZATION OF P. PALMATA POWDER[1]

Introduction

Wild and the artificially cultured strains of P. palmata are highly perishable and bulky when harvested due to their high moisture content (84% w/w). The purpose of dehydration is to increase the shelf life, reduce the bulk weight for easy handling and extract the lipids by supercritical carbon dioxide (SC CO_2). Not only the weight fraction of initial extractable lipids is higher in dried dulse, but also the efficiency of extraction is expected to improve as water will interfere by increasing the polarity of SC CO_2.

It has been reported that the rate limiting step in leaching of organic materials is generally the diffusion within the solid matrix (Schwartzberg, 1980; Segado, 1989). Therefore, it is customary to grind the solid material before extraction to increase the surface area and to decrease the resistance to diffusion of solute in solid-liquid extraction. Size reduction is a common pretreatment before leaching. Since size reduction is an energy demanding operation, the practical limits are determined by the nature of the material and the solute and its concentration.

Dulse contains both polar and non-polar lipids. The polar lipids, i.e. glyco- and phospholipids are generally

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[1] A version of this study has been submitted for publication in the J. Food Process Preserv. (Mishra et al., 1992).

constituents of the cell membrane, while non-polar lipids such as triglycerides and diglycerides function as storage lipids and are distributed randomly inside the cell. The purpose of grinding the dried dulse is, therefore, to increase the surface area for mass transfer and to break the cellular structure to facilitate greater access for extraction of lipids. However, special care must be exercised in grinding dulse, since the polyunsaturated fatty acids (PUFA) are sensitive to heat generated during size reduction.

Physical characteristics influence not only handling (e.g. filling and mixing) and storage behaviour (stability and appearance) of dried dulse, but also the course of extraction by their effect on packing behaviour, heat and mass transfer during extraction, and particle-gas interactions between SC CO_2 and dulse. The extraction of a solute entrapped in a solid matrix is usually carried out in a packed or fixed bed of solids. In a packed bed the supercritical fluid flows through the interstitial spaces of the heterogenous solids that form the bed. Performance of such a contactor depends on the characteristics of the bed of solids and the fluid flowing through the bed. Also, uniform solvent velocity over the cross section of the bed is a prerequisite for minimizing both the amount of solvent and the size of equipment. This is particularly necessary in the case of supercritical fluid technology which is highly capital intensive. Flow instability due to viscosity gradients is known to decrease the

efficiency of a packed bed in solid-liquid extraction of vegetable oil from oilseeds (Spaninks and Bruin, 1980) using liquid solvents. In supercritical fluid extraction (SFE) of solids, poor extraction efficiency results from particle entrainment and channelling of fluid (Toledo, 1991). Both of these problems are related to solid characteristics i.e. particle size, shape and porosity, which are important in the application of SFE.

This chapter describes the physical characteristics of dulse powder in terms of particle, bulk and tapped densities, particle size distribution, compressibility and compaction, and water adsorption behaviour.

Materials and Methods

Cultured strain of P. palmata (100 kg) was received in Edmonton and freeze dried as described in Chapter 2. The freeze dried sample of cultured strain was ground in a Fitzmill portable grinder, model TS6 (Fitzmill Patrick Co., Elmhurst, IL., US) at NORAC Extraction Centre, Edmonton. A 16 knife impact cutter was run at 1300 rpm by an electric motor (5 HP, 480 V) and the material was allowed to pass through a stainless steel sieve with round punched holes. The casing was under cryogenic atmosphere to prevent oxidation and heat degradation of lipids. Temperature of the product was maintained below -50°C during grinding.

The ground material was thoroughly mixed to achieve uniformity of samples and packaged in high density polyethylene bags and sealed under vacuum (Multivac type AG 500, Sepp Haggenmuller KG, Wolfertschwenden, Allgan, Germany). The packages were stored at -75°C.

Determination of physical characteristics

Sieve Analysis

A 50 g sample of dulse powder was sieved through a set of Canadian standard sieves of known mesh size for 30 min in a Tyler Sieve Shaker (Model No. PX 21, The W.S Tyler Company of Canada Ltd., St. Catharines, ON.) at a constant speed of vibration. The sieves used and the corresponding mesh sizes are given in Appendix I (Table I.1). The fraction of the material retained on each sieve was determined by recording the weight of powder after sieving. Sieve analysis was repeated twice.

A mass mean diameter (D_w) of the dulse powder was calculated from the mass fraction of material retained in a given increment of sieve size (X_i) and the particle diameter (D_p), according to the following equation (McCabe et al., 1985):

$$D_w = \sum_{i=1}^{n} x_i D_p \qquad (3.1)$$

Density of dulse powder

Bulk density and tapped density were determined by measuring the volume of known mass of powder. Weighed powder

was carefully poured into the measuring cylinder, which was earlier calibrated by water at 23°C, and the volume was noted. This volume was used in the calculation of bulk density (ρ_b) under free flow.

The cylinder was tapped and the corresponding change in the volume was noted. It was observed that after eight tappings, there was no further change in the volume, and the density corresponding to this volume was reported as tapped density (ρ_t).

The particle or true density was measured by liquid pycnometry (Svarovsky, 1987). The liquid used to displace air was n-butyl alcohol. The particle density was defined as the volume occupied by the known weight of dulse. The exact volume of pycnometers was obtained with water at 23°C (room temperature). The procedure used for the determination of volume of dulse powder was outlined by Svarovsky (1987) for powders.

Compressibility (C) and porosity (ε) of dulse powder were derived from the measured properties i.e. bulk, tapped and particle densities. Compressibility was calculated from tapped and free flow bulk density by the following equation (Svarovsky, 1987):

$$C = \frac{100}{\rho_t}(\rho_t - \rho_b) \qquad (3.2)$$

The porosity of dulse powder was calculated from the particle density (ρ_p) and bulk density using the following equation:

$$Porosity = 1-(\frac{\rho_b}{\rho_p}) \qquad (3.3)$$

The reported values are means of at least 6 determinations.

Water adsorption isotherm

Adsorption isotherm was established by exposing a known mass (5.0 g) of dulse powder in desiccators containing saturated solutions of salts in water (Karel, 1975). The solutions used are given in Appendix I (Table I.2). Saturated salt solutions gave water activities of 0.11, 0.23, 0.32, 0.52, 0.67 and 0.86 (Rockland, 1960). Anhydrous phosphorous pentoxide and water were used as such to represent zero and one water activities, respectively. To avoid mold growth on the samples at higher water activities, a vial containing formalin was placed in the desiccator. The samples were allowed to equilibrate for a period of 2 weeks. Constant sample weight indicated the equilibration of water activity between the sample and the humidity inside the desiccator. Samples were weighed and then dried in a vacuum oven at 80°C overnight. The moisture content was calculated from the weight loss upon vacuum drying. The initial moisture content

of the powder was 6.05% (w/w). The whole procedure was repeated twice.

Results and Discussion

Particle size analysis

Physical characterization of ground dulse was accomplished at two levels i.e. particle and bulk. Microscopic examination of the powder revealed that the particles were irregular in shape and size. No definite shape can be attributed to the particles, which may be regarded as "thin flakes" of variable sizes. Sieve analysis of the powder showed differences in the size as well. The size varied in the range of 137.5 to 462.5 μ and based on the mass of the powder retained on each screen, about 90 % of the particles passed <400 μ size (Figure 3.1). Mass mean diameter of the particles calculated from such an analysis was 208 μ.

Physical characteristics

Physical characteristics of dulse powder depend on the physicochemical nature of the particle and the process used in their formation. These characteristics are given in Table 3.1. Measured values are for a powder having a mean moisture content of 6% (w/w). The particle density was 1674 kg/m^3, which compares well with the reported composite densities of major constituents of dulse, i.e. cellulose, salt and proteins (Peleg, 1983).

Powder rheology depends on the properties of the bulk of particulated bed. This means that the macroscopic structure of the arrangement of particles and their shape are extremely important in determining rheological properties of the powders. Increase in bulk density after tapping from 343.6 to 420.6 kg/m^3 indicated porous nature of powder. The calculated porosity of the bulk of 0.79 and a compressibility of 18% suggest that a bed of dulse powder would compress under applied stress, which is very important in packing behaviour. Moreover, due to the inherent anisotropy of powder, the pore size would be expected to vary.

The compaction characteristics of dulse powder were evaluated by studying the relationship between the reduction in volume upon vertical tapping and the number of taps (n). The following relationship has been proposed by Sone (1972):

$$\gamma_n = \frac{V_o - V_n}{V_o} = \frac{abn}{1+bn} \qquad (3.4)$$

Peleg (1983) linearized eq (3.4) to the following form:

$$\frac{n}{\gamma_n} = \frac{1}{ab} + \frac{n}{a} \qquad (3.5)$$

A plot of the left hand side of eq. (3.5) versus n yields a straight line with a slope and intercept equal to 1/a and 1/ab, respectively. Such a plot was constructed for dulse powder and silica gel desiccant (Figure 3.2). The difference between the two was apparent. The values for constants a and

b for dulse were 0.038 and 14.88 in comparison to 0.024 and 6.21 for silica, respectively. Compared to silica, dulse powder was more cohesive and plastic requiring a large number of vibrations for compaction. A large part of interparticle porosity in dulse powder is due to the structural voids supported by the forces of van der Wall's type (Peleg, 1977). Being weak in nature, these forces will collapse under even a small stress, consequently increasing the bulk density. Although this vibration test does not conclusively predict compaction, it leads to a speculation that a bed consisting of dulse powder may compact under applied stress. The irregularly shaped particles may change their orientation and interlock to form a mechanically stable structure with variable porosity. All of these properties are affected by moisture content. Moisture in the powder cements the particles together by formation of 'liquid bridges' (Peleg, 1983). As a result, the bulk density is increased. These bridges are responsible for caking of powders.

Sorption isotherm

Water plays a major role in influencing various properties of foods. However, it is the state of water that is more important than the moisture content itself. Sorption isotherms relate moisture content of product to its water activity (a_w), a property of utmost significance in microbial and lipid stability of dulse during storage and processing. At high a_w, it is impossible to extract lipids by a non-polar

solvent like SC CO_2. A_w may also influence extraction by releasing lipids bound to cell membranes by breaking lipoprotein linkages.

Figure 3.3 shows the adsorption isotherm of dulse at 23°C, which conforms to a Type II isotherm according to Brunauer's classification (Labuza, 1984). There was little increase in the moisture content of the dulse powder until the a_w reached about 0.6, after which a sharp increase in the moisture uptake was observed. This behaviour is characteristic of sorption isotherms for crystalline materials like salts or sugars. These components in dulse must have crystallized and become concentrated during freeze dehydration. High hygroscopicity was observed at $a_w > 0.8$ as amount of moisture absorbed rose sharply from 63.6 g water/100 g dry solids at a_w of 0.86 to 132 g water/100 g of dry solids at a_w of 1.

The form of BET equation applied to the adsorption data is (Labuza, 1968):

$$\frac{a_w}{(1-a_w)X} = \frac{1}{X_m C} + \frac{C-1}{X_m C} a_w \qquad (3.6)$$

A plot of the left hand side of eq. (3.6) as a function of a_w yielded a straight line in the range of water activity <0.4 (Figure 3.4). From the slope and intercept of this line, X_m and C were calculated. The surface area of the powder (A_s)

was calculated assuming a surface area of water molecule to be 10.6 x 10^{-20} m^2 by the following equation (Labuza, 1968):

$$A_s = X_m N \frac{A_{wat}}{18} = 3.5x10^3 X_m \qquad (3.7)$$

Table 3.2 shows the calculated values of BET parameters. The monolayer value of 4.8 kg/100 kg dry solids is in agreement with a reported value of 4.92 kg/100 kg dry solid for dehydrated fish (Labuza, 1984). Relatively high values for the constant C indicate more polar sites for water binding, which resulted in a high value for heat of adsorption. The BET surface area was 169 m^2/g which compares well with 165 m^2/g for freeze dried salmon (Labuza, 1968). It should be pointed out that the surface area calculated from water adsorption differs appreciably from the values obtained with nitrogen adsorption. This is due to the smaller size of water molecule as compared to nitrogen and the plasticizing action of water on the polymers that form the structural matrix of the material. Swelling resulting from the adsorption of water usually increases the surface area by exposing more polar sites, reducing the accessibility of lipids for extraction by SC CO_2.

The adsoption isotherm at a_w<0.5 fits the BET sorption model. The data for a_w>0.5 were fitted with Harkins-Jura-Smith model (Lang and Steinberg, 1980). The best fit equation (R^2= 0.98) for adsorption of water for dulse powder at 23°C was:

$$X = -23.67-45.734\ln(1-a_w) \qquad (3.8)$$

Results of adsorption isotherm indicate that dulse powder is extremely hygroscopic due to high salt content. The powder will have a tendency to form cakes during storage, handling and extraction. Caking may reduce extraction efficiency primarily by improper flow distribution of SC CO_2. Due to high moisture contents at a_w>0.5, the extraction efficiecy of lipids will be poor due to reduced activity of lipids in powder, which in turn will reduce their solubility in SC CO_2. The overall effect of a_w on the behaviour of lipid extraction will be a net result of modification of solvent polarity and/or influence on powder-water-lipid interactions. It has been reported earlier by McHugh and Krukonis (1986) that it was impossible to extract caffeine from coffee beans by either dry SC CO_2 or methylene chloride. Moisture was necessary for extraction. As caffeine is, indeed, soluble in dry SC CO_2, it was reasoned that dry SC CO_2 could not break the caffeine-coffee bean bonds and water was required to free caffeine from the solid matrix. In the case of dulse, presence of water may release some of the membrane bound lipids, thereby increasing solubility in the lower range of a_w (<0.5). Therefore, it appears that there should be an optimum a_w corresponding to a maximum recovery of lipid.

Conclusion

Based on the data on the physical properties, the dulse powder can be characterized as irregularly shaped, porous material with a low bulk density. The mean particle diameter was 208 micron. The vibration test indicated cohesive nature. Such a product may cake and become compacted under applied stress. Due to its fine particle size, particle entrainment will pose a problem, particularly at high solvent flow rates. High porosity and compressibility of the powder may offset the benefits derived from reduction of particle size to improve extraction efficiency by increasing the surface area for mass transfer.

Water adsoption behaviour was established for dulse powder. Water activity below 0.5, can be predicted with BET model, while Harkin-Jura-Smith model was proposed to explain the dependence of a_w on the moisture content at a_w levels higher than 0.5. The sorption pattern indicates that dulse powder is hygroscopic with a BET monolayer value of 4.8 kg water adsorbed/100 kg of dry dulse. In order to ensure prevention of caking and oxidative degradation of lipids, the powder should be kept near this monolayer moisture during storage. Water may play a role in increasing the total solubility of lipids during SFE by influencing the polarity of SC CO_2 and releasing the lipids bound to the solid matrix. Net effect may be an increase in the total solubility of

lipids due to extraction of some polar lipids with neutral lipids.

Table 3.1. Physical characteristics of P. palmata powder.

Characteristics	Value
Bulk density (free flow)* ρ_b (kg/m^3)	343.6
Tapped density* ρ_t (kg/m^3)	420.6
Particle density* ρ_p (kg/m^3)	1674.8
Porosity, e	0.795
Compressibility, C (%)	18.3
Moisture content (% w/w)	6.0

* The data are means of 6 analyses.

Table 3.2. BET parameters of water adsorption for P. palmata powder at 23°C.

Parameter	Value
Monolayer moisture X_m (kg/100 kg dry solid)	4.8
C	31.7
BET area A_o (m^2/g solid)	169.0
Heat of adsorption* H (kJ/kg)	472.8

* calculated from BET constant C.

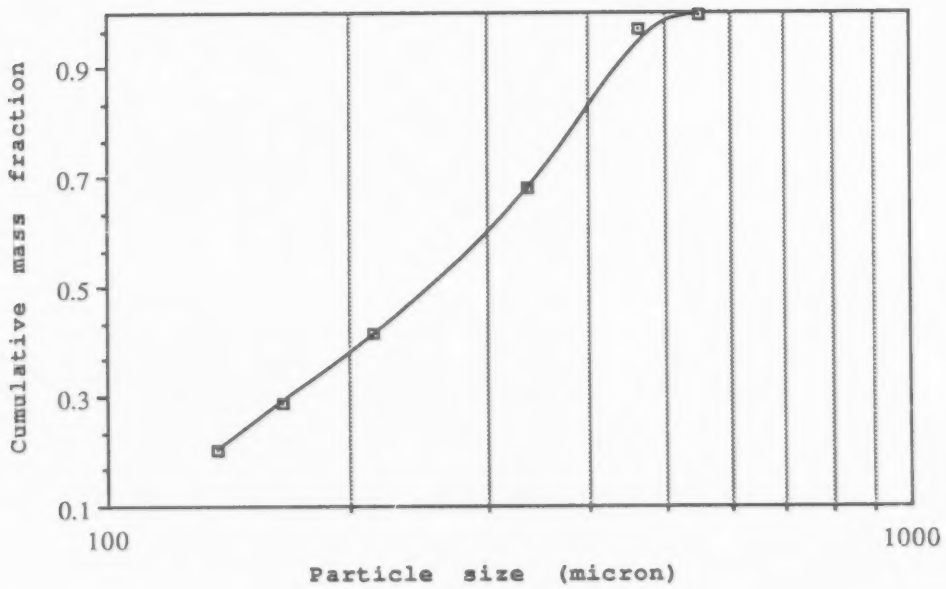

Figure 3.1. Cumulative screen analysis of dulse powder.

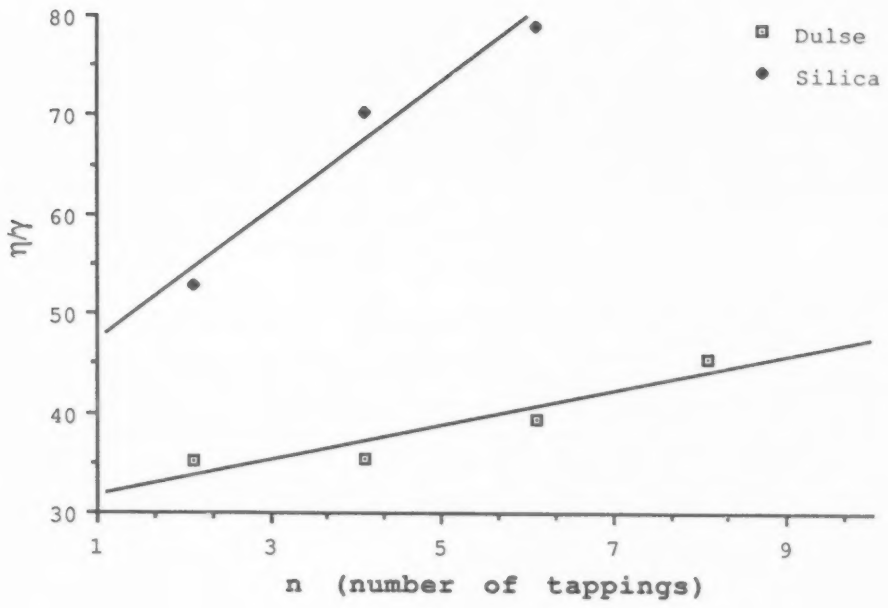

Figure 3.2. Effect of tapping on the change in volumes for dulse and silica powder.

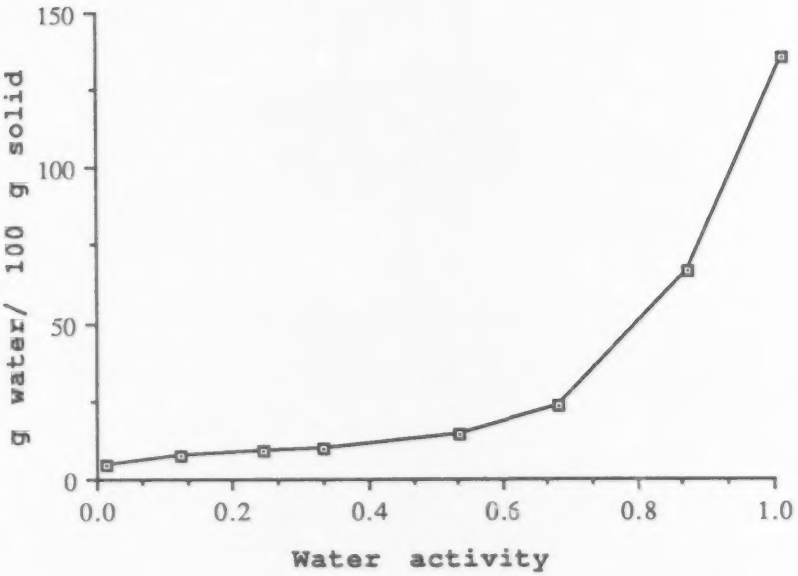

Figure 3.3. Adsorption isotherm of dulse powder at 23°C.

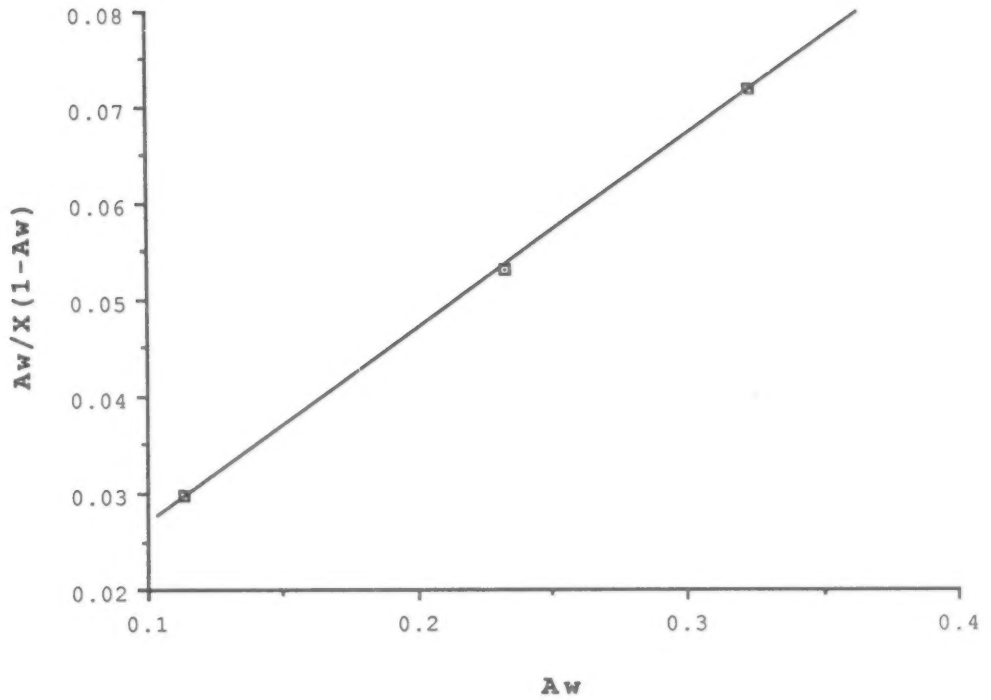

Figure 3.4. BET plot of adsorption data for dulse powder at 23°C.

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4. SUPERCRITICAL CARBON DIOXIDE EXTRACTION OF OIL FROM P. PALMATA[1]

Introduction

The omega-3 (ω-3) fatty acids have been found to have potential health benefits in various inflammatory diseases e.g. atherosclerosis, asthma, arthritis and cancer (Weaver and Holub, 1988). Among these polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3) are of major importance due to their immediate effects on plasma lipids (Kinsella, 1990). Currently, major source of ω-3 fatty acids is fish oils. The ω-3 fatty acids content of fish oils can be as high as 25%. However, there is a considerable variation in the composition of fish oils depending on the species, parts of the fish, and location of the catch, etc. Since the demand for ω-3 fatty acids is expected to increase, which may not be met by fish oil alone, alternative sources of these fatty acids are currently being sought to fulfil the demand (Yongmanitchai and Ward, 1989).

Supercritical fluid extraction (SFE) is emerging as an important separation method in the field of food and pharmaceutical applications. With this technology it is possible to separate heat sensitive compounds like ω-3 fatty acids and to avoid any toxic solvent residues in the product. The isola-

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tion and fractionation of ω-3 PUFA, using supercritical carbon dioxide (SC CO_2), from fish, fish oil and esters have been studied by several researchers (Eisenbach, 1984; Yamaguchi et al., 1986; Nilsson et al., 1988, 1989; Rizvi et al., 1988). Eisenbach (1984) used SC CO_2 at 15.2 MPa/50°C to fractionate ethyl esters of cod fish oil. A fraction containing C20 esters in 90% purity was achieved with a hot finger. Rizvi et al. (1988) compared the yield of EPA from various feedstocks i.e. free fatty acids, esters of fatty acids and fish oil and showed that fatty acid esters had the highest solubility in SC CO_2. The concentration of ω-3 fatty acids was increased from 40% to about 88% using a refluxing system and a feed of free fatty acids. Nilsson et al. (1988) reported 96% concentration of ω-3 fatty acids from urea pretreated menhaden oil fatty acid ethyl esters by a temperature gradient in a column at 15.16 MPa. Incremental pressure programming (13.1-15.1 MPa) at a maximum temperature of 80°C (Nilsson et al., 1989) was also used to fractionate the same feed material.

It has been shown by many researchers that addition of a suitable cosolvent or entrainer in a supercritical fluid increases the solubility and selectivity of a solute significantly in SC solvents (Brunner and Peter, 1982; Walsh et al., 1987 and Ikushima et al., 1988). The possibility of achieving increased solubility at lower extraction pressures adds to the economical advantage gained by the saving in energy and equipment costs. By increasing the temperature dependence of

solubility with entrainers, it is possible to effect separation of solutes from the supercritical solvents by isobaric increase or reduction in temperature resulting in further savings in energy.

Yamaguchi et al. (1986) were the first to extract fish oil enriched in EPA directly from krill meal and freeze dried krill. At 25.4 MPa and 80°C the recovery of oil was 11.5 g/100 g from freeze dried krill and 4.0 g/100 g from krill meal. Hardardottir and Kinsella (1988) used SC CO_2 at 13.78-34.46 MPa and 40-50°C to extract lipid and cholesterol from trout muscle. The use of algae for the extraction of ω-3 oils is relatively new. Polak et al. (1989) studied SC CO_2 extraction of lipids from freeze dried microalgae Skeletonema costatum (25% EPA) and Ochromonas danica (11% EPA) at 17-31 MPa and 40°C.

Palmaria palmata of the class Rhodophyta was selected as a raw material for extraction of oil because of its very high EPA content (45%) on the total fatty acid wt basis and very high percentage in C20 fraction (Mishra et al., 1992). The objective of this work was to test the feasibility of extraction of lipids by SC CO_2 from P. palmata at different extraction temperatures and pressures under equilibrium conditions and analyze the extracts for fatty acid composition with an emphasis on EPA.

Materials and Methods

Material

Palmaria palmata used in this study was cultured under controlled laboratory conditions at the National Research Council, Halifax, NS. The conditions used for the cultivation and freeze drying have been described in Chapter 2. The material was ground, vacuum packaged and stored as described in Chapter 3.

Extraction system

The supercritical extraction unit was manufactured by Newport Scientific Inc. (Jessup, MD). Carbon dioxide (99.5% purity) was compressed to the desired pressure by a diaphram compressor with a maximum rating of 69 MPa. The extraction vessel (300 mL, 300SS) was heated by two silicon-fibreglass electrical resistance type heating tapes (100W each), with a burnout temperature of 232°C and temperature was controlled by a thermostat (±1°C) via a type J thermocouple immersed at the centre of the vessel. Pressures at the compressor suction and discharge (i.e. extraction vessel) were measured through two bourden tube gauges. The extraction pressure was controlled by a back pressure regulator (Tescom Co, Elk River, MN). A rupture disc was provided as pressure overshoot protection. The system was equipped with a hand operated needle valve for depressurization of SC CO_2. The valve was heated to avoid freezing due to expansion of SC CO_2. The extract was collected in glass tubes placed in a refrigerated bath (Frigo Plus, B.

Braun, Melsungen, Germany). Flow rate of the gas passing through the extraction vessel was controlled by periodic adjustment of this expansion valve. The depressurized CO_2 was passed through a rotameter and the volume of the gas at the ambient temperature and pressure was recorded by a dry testmeter (American Meter Co.) before venting to the atmosphere.

The SCE system was slightly modified for the current investigation. It was difficult to completely seal off leaks at the vessel head with the original metal gasket, hence, a Buna O-ring was placed at the top etched end of the vessel. This arrangement provided complete sealing by the expansion of the o-ring. A stainless steel basket that fits into the extraction vessel was fabricated to facilitate loading and unloading of the sample. The ends of the basket were mounted with two 10 μ metallic filters (Mott Metallurgical Corporation, Farmington, CT), to contain the sample and to avoid carry over of the particles. In order to prevent the contamination of rotameter and flow totalizer with any residual volatiles entrained in CO_2, a bed of silica gel was placed between the expansion valve and the rotameter. The salient features of the system are depicted in Figure 4.1.

Extraction procedure

Fourty grams of throughly mixed ground sample was contacted with upflowing SC CO_2 at the set temperature and pressure in the extraction cell. CO_2 gas at the tank pressure

(3.5-7.0 MPa) was allowed to fill the extraction vessel, while keeping the expansion valve closed. The compressor was switched on and the system was allowed to reach the desired pressure by actuating the back pressure regulator. Once, the desired pressure and temperature were achieved, the system was allowed to equilibrate for 15 min. The expansion valve was slowly opened and the flow rate was adjusted to desired level. The extract was collected in glass tubes maintained at -20°C in a refrigerated bath using ethylene glycol. The extracts were stored at -75°C.

The pressure and temperature ranges studied were 20.68, 41.36, 62.15 MPa and 35, 45, 55°C, respectively. Solvent flow rate was maintained at 300 mL/min in order to approach equilibrium conditions (McHugh and Krukonis, 1986), by continuous adjustment of the expansion valve. The extracts were weighed periodically and the corresponding volume of CO_2 passing through the cell was recorded by a dry gas meter at ambient conditions. The solubilities were calculated from a plot of the amount of total lipid recovered versus the corresponding volume of CO_2 used at ambient conditions. Extractions under each temperature and pressure condition were repeated twice and the means of the two determinations were reported.

In a separate experiment, lipids from dulse were extracted with chloroform-methanol according to the method of Bligh and Dyer (1959) as given in chapter 2 and infused in

alumina beads. The beads were then extracted with SC CO_2 at 62.15 MPa and 45°C. Lipids recovered from alumina beads were compared to those extracted from seaweeds under similar conditions. The fatty acid composition of five sequential lipid fractions, collected over every hour through a five hour period from the alumina beads, was determined. Extraction of lipids from dulse, infusion in alumina beads and SC CO_2 extraction of beads were repeated twice.

The system lacked the provision for continuous addition of a cosolvent directly into SC CO_2. Hence, to study the effect of a polar entrainer on EPA recovery, ethanol was mixed with the seaweed (10% w/w) in an air tight container and allowed to equilibrate overnight. Seaweed pretreated with ethanol was extracted with SC CO_2 at 41.36 MPa and 45°C.

Analysis of extracts

The extracts were transmethylated to fatty acid methyl esters (FAME) and were analyzed by gas chromatography (GC) as described earlier (Chapter 2). Triplicated GC injections were made on every lipid extract obtained from SC CO_2 extraction.

Lipid classes in the extracts obtained by chloroform-methanol and SC CO_2 (20.68 MPa and 45°C) with and without ethanol as cosolvent were determined by thin layer chromatography (TLC) (Christie, 1982). Lipid samples were applied on precoated silica gel-G plates (250 μ thickness) and the classes were separated in a solvent system consisting of hexane, diethyl ether and formic acid in the proportions of

80:20:1, respectively. The plates were sprayed with sulphuric acid (50%) and heated in an oven maintained at 200°C for 40 min. Individual classes were identified by comparison with the characteristic RF values for standards.

Results and Discussion

The solubility of oil from P. palmata under different extraction conditions is shown in Table 4.1. The results show that the solubility varied from a minimum of 2.88 mg/L at 41.47 MPa/45°C to 3.63 mg/L at 41.47 MPa/55°C. There was no difference in the solubilities of total lipids under these conditions. This can be explained on the basis of the composition of the extracts obtained at different extraction pressures and temperatures. The lipids of P. palmata were found to be a mixture of triglycerides, free fatty acids, sterols, hydrocarbons and phospholipids (Chapter 2). These components differ in their molar mass, vapor pressure and polarity, which determine solubility behaviour in SC CO_2. For example, triglycerides are preferentially soluble in SC CO_2 than phospholipids.

Due to the small amounts of the extracts, it was not possible to determine the exact composition of each of the lipid components, which otherwise could have ascertained the effects associated with component selectivity. Even though there may be significant differences in solubilities of individual lipid classes as a function of extraction condi-

tions, differences in total lipid solubility were found to be insignificant within the temperature and pressure ranges studied. For most solutes studied, an increase in extraction pressure resulted in an increase in solubility due to an increase in CO_2 density (McHugh and Krukonis, 1986). However, the fact that an increase in pressure did not always translate into an increase in solubility was reported in earlier studies (Schmitt and Reid, 1986; Yamaguchi et al., 1986; Hardardottir and Kinsella, 1988; Polak et al., 1989; and Vijayan and Buckley, 1991). Polak et al. (1989) reported no significant difference in the solubility of lipids at 24 and 31 MPa for microalgae. Vijayan and Buckley (1991) found that the solubility of lipids from potato chips was higher at 41.47 MPa than that at 55.15 MPa. No explanation was extended for the results.

Another contributing factor may be the hydrodynamic instability in the extraction column leading to channelling (Spaninks and Bruins, 1980). Particle entrainment and channelling have been reported to decrease extraction efficiencies in SFE (Toledo, 1991) and leaching operations. Poor contact between the solvent and seaweed may have resulted in a low recovery of lipids. Although glass beads were mixed with seaweeds, a significant degree of packing of the bed was noticed upon opening the cell after each run. In a separate experiment at 62.15 MPa/45°C, after a five hour contact time, the bulk density profile of the bed was measured by noting the

change in the volume of the bed. Considerable caking of bed was noticed. The measured bulk density of the caked portion of bed was 510.8 kg/m^3 as compared to 351.6 kg/m^3 for the bed before starting the experiment. This certainly suggested caking during the extraction run. With the present experimental setup, it was not possible to quantitate the effects of bed compaction on the efficiency of extraction. However, anomalous solubility behaviour suggests a possible role. Bed compaction and flow maldistribution causing poor extraction efficiency have been noticed for various feed materials e.g. egg powder and yolk (Nguyen, 1992; Novak et al., 1991).

The EPA concentrations of the extracts were also given in Table 4.1. The results indicated higher recovery of EPA at low pressure (20.78 MPa) than at higher pressures. This may be again due to higher selectivity for EPA containing esters at low pressure, as selectivities are inversely related to the density of the solvent (Prange and Riepe, 1987).

Possibility of absorption of some lipids on the solid matrix also cannot be overruled. Upon extraction of lipid infused alumina beads there was a difference in the extraction behaviour, as depicted in Figure 4.2. The recovery of lipids from beads at 60.15 MPa/45°C was higher (4.70 mg/L) than that from seaweed (3.74 mg/L). This suggests that some of the lipids may be in the bound form in seaweed, thereby making it difficult to extract with SC CO_2.

The GC analysis of the fatty acid profile of the sequential fractions is reported in Table 4.2. The fractions represent the hourly collections of the extract from alumina beads. Since myristic (14:0), palmitic (16:0), and eicosapentaenoic acids (20:5, ω-3) constitute >90% of the total fatty acids, the concentrations of only these fatty acids are reported. In all of the five fractions the dominant fatty acid was EPA which is consistent with the overall fatty acid profile of the alga. The first fraction showed the least concentration of the three fatty acids, indicating that the majority of the extracted components were hydrocarbons and/or sterols. The concentrations of the fatty acids in the extract decreased with time after reaching a maximum at 2 hours for 14:0 and 16:0 and 4 hours for EPA, possibly due to the depletion of these fatty acids in the sample.

Eicosapentaenoic acid in P. palmata is distributed in both neutral and polar lipid classes (Chapter 2). SC CO_2 is essentially a non-polar solvent. Hence, the EPA concentration achieved by SC CO_2 extraction represented only the EPA present in the neutral lipids. It was expected that an increase in the recovery of polar lipids together with the non-polar fraction should increase the total concentration of EPA in the extract. For this purpose, a polar cosolvent, ethanol, was added to the system. After completion of the extraction run, the extract was purged with N_2 to remove ethanol. Due to

addition of ethanol (10% w/w) as an entrainer in the seaweed, the total solubility of lipids increased to 9.35 mg/L CO_2 at 20.78 MPa/45°C. Since it was difficult to remove entrained ethanol in the extract completely, this figure may not represent true solubility. The extract was of light green color due to the presence of chlorophyll pigments. TLC of the extract obtained after addition of ethanol confirmed the presence of polar lipids (phospholipids) which were otherwise absent in the extract obtained by SC CO_2 alone under similar conditions of extraction (20.78 MPa/45°C). This effect was, indeed, expected as ethanol increases the polarity of solvent, thereby increasing the solubility of polar components. The EPA content of the extract increased slightly from 2.29 mg to 2.57 mg by ethanol treatment. Table 4.3 provides a comparison of the fatty acid profiles of lipids extracted with and without addition of ethanol at 20.68 MPa/45°C. By addition of ethanol, there was an increase in the combined concentration of saturated fatty acids, i.e. 14:0, 16:0 and 18:0 (20.8% to 25.6%) and of 18:1 (ω-7 and ω-9) (5% to 9.2%). The concentration of EPA in the total fatty acids extracted at 20.78 MPa/45°C/ethanol moderately increased from 56% to 64.1%. It was shown by Nilsson et al. (1991) that addition of 5% ethanol in SC CO_2 enhanced the partition coefficients of all the fatty ethyl esters from menhaden oil by a factor of 1.5-3.1.

Conclusion

It was technically possible to extract lipids from P. palmata using SC CO_2. Based on the solubility results it is not desirable to use pressures higher than 20.78 MPa, which is economically beneficial. A portion of the lipids in P. palmata may be in bound form leading to poor recovery of extract. Addition of ethanol not only leads to an increase in the overall solubility of lipids, coextraction of polar lipids also increases the concentration of EPA in the extract.

Even though P. palmata contains a relatively high initial concentration of EPA (45%), low total lipid content and poor recovery of EPA by SC CO_2 extraction make the economic feasibility of the process based on this raw material questionable. If it is possible to increase the total lipid content by suitable genetic manipulation, this alga can become a potential source of EPA.

Table 4.1. Solubility of seaweed oil and EPA content of the lipid extracts obtained under different operation conditions.

Pressure (MPa)	Temperature (°C)	Solubility* (mg/L)	EPA content (% lipid)
20.78	35	3.47	0.59
	45	3.27	1.04
	55	3.37	0.78
41.47	35	3.26	0.27
	45	2.88	0.23
	55	3.63	0.16
62.15	35	3.37	0.19
	45	3.28	0.06
	55	3.33	0.18

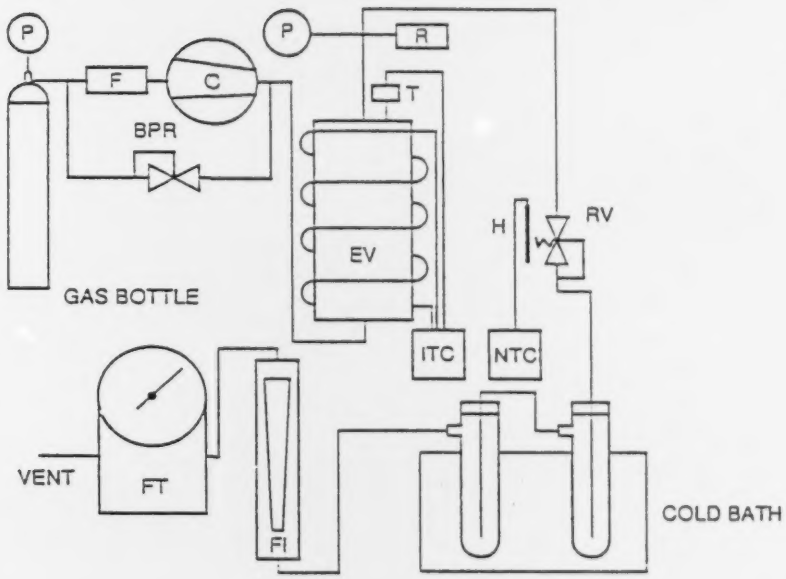
* measured at room temperature and atmospheric pressure

Table 4.2. Fractional recoveries of major fatty acids from alumina beads at 62.15 MPa and 45°C.

Fraction	Weight of extract (mg)	Fatty acid (%lipid)		
		14:0	16:0	20:5ω-3
1	102	0.09	0.47	1.32
2	87	0.43	1.88	5.18
3	121	0.38	1.79	6.04
4	60	0.33	1.39	6.04
5	71	0.27	0.99	2.98

Table 4.3. Effect of ethanol on the fatty acid profile of the lipids extracted by supercritical CO_2 at 20.78 MPa and 45°C.

Fatty acid	With ethanol (wt%)	Without ethanol (wt%)
14:0	3.3	4.2
16:0	15.7	18.3
16:1ω-5,ω-7	4.5	4.9
18:0	1.8	3.1
18:1ω-9,ω-7	5.0	9.2
18:2ω-6	1.3	1.3
18:4ω-3	1.4	tr
20:4ω-6	2.8	3.0
20:5ω-3	64.1	56.0



T	: THERMOCOUPLE	ITC	: INDICATING TEMPERATURE CONTROLLER
F	: FILTER	FT	: FLOW TOTALIZER
H	: HEATER	C	: DIAPHRAGM COMPRESSOR
R	: RUPTURE DISC	EV	: EXTRACTION VESSEL
RV	: RELIEF VALVE	FI	: FLOW INDICATOR
NTC	: NONINDICATING TEMPERATURE CONTROLLER	P	: PRESSURE GAUGE
		BPR	: BACK PRESSURE REGULATOR

Figure 4.1. Schematic diagram of supercritical extraction apparatus.

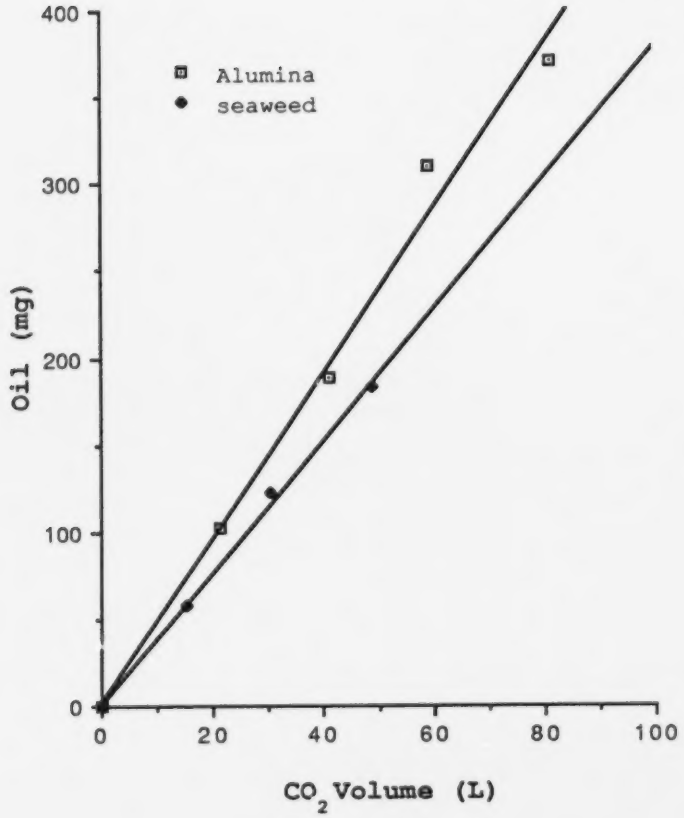


Figure 4.2. Effect of CO_2 volume on the yield of oil extracted from alumina and seaweeds at 62.15 MPa and 45°C.

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5. VAPOUR PRESSURE OF FATTY ACID ESTERS : CORRELATION AND ESTIMATION[1]

Introduction

The design and development of any separation process require knowledge of physicochemical properties of the mixture components to be separated. The properties needed for supercritical fluid extraction (SFE) system may be classified as those of solute, solvent and their mixture. The pure component properties of solute and solvent that directly influence the course of extraction are molar mass, vapour pressure and polarity. These three properties should be either measured, obtained from literature based on experimental data, or predicted from available theoretical models to be able to develop extraction processes.

There is extensive experimental data available on the properties of carbon dioxide, a supercritical solvent of choice for biomaterials. However, the data for the properties of fatty acids and their esters are not only sparse, but also quite variable (Holman and Rahm, 1966). Vapour pressures and enthalpy of vapourization for these compounds are available mostly for saturated short chain fatty acids and their esters (Formo, 1979). The data for polyunsaturated fatty acids (PUFA) and esters of biological importance are lacking. Such

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[1] A version of this study has been submitted for publication in J. Food Eng. (Mishra et al., 1992).

data are not only valuable in traditional separation methods e.g. distillation and solvent extraction but also important in SFE.

The prediction of equilibrium solubilities in supercritical solvents has been attempted by employing equations of state (Vetere, 1979; King and Bott, 1982; King et al., 1983; Kwak and Mansoori, 1986; Rizvi et al., 1986; 1988), and solution thermodynamics (Kurnik and Reid, 1982; Mansoori and Ely, 1985). These models require an extensive database on the physical properties of solutes i.e. critical temperature, critical pressure, critical volume, and acentric factor. Since there are no experimental data available on these properties of fatty acids and their esters, it is common to estimate them using group contribution techniques (Reid et al., 1987). Group contribution methods for estimation of critical temperature often require experimentally determined normal boiling point (NBP) as a parameter. In the dearth of experimental data on NBPs of fatty acids and their esters, NBP also need to be estimated. Therefore, errors introduced in the estimation of NBPs will have a cumulative effect on corresponding critical properties, resulting in failure of the model.

Traditional methods for measurement of vapour pressure and enthalpy of vaporization by calorimetry or ebulliometry are less accurate for the low boiling compounds such as PUFA. The discrepancy in the literature values for these properties

is related to the uncertainty involved in measuring pressure. Due to high boiling nature of fatty acids and their esters, high temperatures and high vacuum are required to measure vapour pressures. Use of high temperatures in an experimental protocol may lead to heat decomposition and oxidative degradation of ω-3 fatty acids with high degree of unsaturation. This necessitates the use of non-equilibrium techniques which are limited in their use for being time consuming.

Gas-liquid chromatographic retention data on non-polar stationary phases have been related to solute vapour pressures (Purnell, 1962; Ambrose, 1971; Conder and Young, 1979). Advantages of gas liquid chromatography (GLC) for the determination of physicochemical properties of fatty acids and their esters are as follows:

1. It can be used for high boiling unsaturated solutes, where other methods can not be used.
2. It requires a small amount of sample.
3. The sample need not be pure.
4. GLC being a common analytical technique, the instrument is readily available.
5. Reproducible retention data can be generated rapidly.

In the present study Meissner's group contribution method (Perry and Chilton, 1973) of predicting NBPs of fatty acids was evaluated. In the second part, retention data from GLC were used to estimate vapour pressure and enthalpy of vaporiz-

ation of fatty acid esters. Correlations based on above data are proposed.

Materials and Methods

Pure fatty acid methyl and ethyl esters were purchased from Sigma Co., Montana. The esters were dissolved in n-hexane to get a concentration of 100 µg/mL for injection to a gas chromatograph.

Gas liquid chromatography

A Varian Vista 6000 gas chromatograph (Varian, Palo Alto, CA) equipped with a flame ionization detector, an integrator and an autosampler was employed for measurement of retention data. Ultra high purity grade helium was used as a carrier gas. Gas flow rate was measured at 30°C by a soap-bubble flow meter. Column inlet pressure was 0.138 MPa. Injector and detector were maintained at 250°C and 270°C, respectively, during the run. Three injections were made to measure retention times for each fatty acid ester (FAE). The retention times for each FAE were corrected by substracting the retention times for methane and n-hexane at identical experimental conditions.

A fused silica capillary column (12 m x 0.22 mm internal diameter, SGE Inc., Austin, TX) was used for the determination of retention times. The non-polar stationary phase was BP 1, which is a polymer of dimethyl siloxane. This phase has been selected for these experiments due to its ideal thermal and

chemical stability to prevent oxidative degradation of PUFAs e.g. eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and due to the fact that separation on BP 1 stationary phase is based on the difference in boiling points (Bruno and Svoronos, 1989). The amount of liquid phase in the column varied between 7.2 to 10.4 mg for the column used (SGE Inc., Austin, Texas). In the calculation of specific retention volume an average value of 8.9 mg was used.

Theory

In gas-liquid chromatography, elution of a sample from a column depends on the relative affinity of the solute to the mobile and liquid stationary phases. The relative affinity of a solute is generally expressed in terms of distribution coefficient (K), which is defined as the ratio of the concentration of solute in liquid to that in mobile phase. This coefficient depends on the vapour pressure of pure solute and the extent of its interaction with the liquid phase. It provides the theoretical basis for calculation of equilibrium properties such as vapour pressure and activity coefficients of pure solute from the readily measured chromatographic retention parameters, i.e. the retention volume.

The retention volume of a solute (V_r) at a given set of conditions of gas chromatography is given as:

$$V_r = t_r F_c \qquad (5.1)$$

This parameter is corrected by the retention time of unretained sample e.g. methane, to account for the column dead volume. Equation (5.1) can be written as net retention volume of the solute (V_n) as

$$V_n = (t_r - t_{ra}) F_c \qquad (5.2)$$

Both of these parameters vary with the experimental conditions and the amount and nature of the stationary phase. It is customary, therefore, to express V_n per unit weight of the stationary phase at 0°C. The resulting expression (eq. 5.3) gives specific retention volume (V_s), a characteristic of the solute and stationary phase,

$$V_g = V_n \frac{273.15}{W_1 T} \qquad (5.3)$$

The relation between specific retention volume (V_s), vapour pressure of solute (p^o) and activity coefficient (γ) could be derived from the thermodynamics of chromatographic separation (Purnell, 1962). In the simplest case, the relationship is given as:

$$V_g = \frac{273.15\ R}{p^o M_1 \gamma} \qquad (5.4)$$

The derivation of eq. (5.4) can be found in standard text books on gas chromatography (Purnell, 1962; Conder and Young, 1979). Equation (5.4) forms the basis for calculation of vapour pressures of fatty acid esters from the GLC retention

data provided the stationary liquid phase and the solute form an athermal solution.

Results and Discussion

Estimation of normal boiling points using Meissner Equation

Thermodynamic models used in supercritical fluid extraction need a prior knowledge of critical properties. For fatty acids and their esters, these properties are not available in literature, and the usual approach is to use group contribution techniques for estimation (Bharat et al., 1989; Liong et al., 1992). Most of these methods use experimental boiling points as a parameter in the estimation of critical temperature. In the absence of experimental data on NBPs of PUFA, this property has to be estimated by group contribution techniques (Reid et al., 1987; Perry and Chilton, 1973). Meissner (1949) proposed the following relationship between the normal boiling point, molar refraction (R_D), parachor (P) and a constant, characteristic of chemical type:

$$T_b = \frac{637\,[R_D]^{1.47}+B}{P} \qquad (5.5)$$

Meissner's equation gives an average absolute error of 2% (Perry and Chilton, 1973). Since both R_D and P are not readily measured, these properties are evaluated by summation of contributions for the constituent atoms and structures. In this study, R_D and P were estimated from the structural contributions given by Perry and Chilton (1973). A computer

program based on structural contributions and a constant B in Meissner equation was written as a part of this study and used to calculate boiling points. The program was specifically written for the estimation of normal boiling points of fatty acids and their methyl and ethyl esters. A comparison of estimated and experimental boiling points for selected fatty acids and methyl myristate is given in Table 5.1. The experimental NBPs for fatty acids and methyl ester of myristic acid were taken from Singleton (1960) and Perry and Chilton (1973), respectively. Equation (5.5) underestimated boiling points for the fatty acids used in the study. Absolute error varied from 1 to 5.6% for fatty acids. An upper and a lower range of error were found for myristic acid and linoleic acid, respectively. Error of estimation increased with decreasing chain length for saturated fatty acids and decreased with increasing degree of unsaturation. Also, the reduction in error of estimation for methyl myristate as opposed to that of myristic acid suggest better prediction capabilities of the method for esters. Equation (5.5) failed to predict a decrease in boiling point by introduction of double bonds, e.g. the estimated boiling point of oleic acid was higher than that of stearic acid. Since it is based on group contribution, the method will predict similar NBPs for two positional isomers of PUFA, which is a weakness of Meissner scheme.

Error in the estimates may be in part due to the fact that the group contribution values used in the determination

of R_D and P vary with chain length of fatty acids. Fuchs and Peacock (1980) showed that contribution due to methylene increments to the standard enthalpy of vaporization was not constant and varied from 4.27 to 5.31 kJ/mol for C5-C15 fatty acid methyl esters. Incorporation of variations in group contribution may improve the boiling point estimates, however, such a treatment will result in a loss of simplicity of the group contribution method.

Estimation of vapour pressures from GLC data

Table 5.2 shows V_s data as a function of temperature for selected methyl and ethyl esters of fatty acids. The effect of carrier gas compressibility on V_s was accounted for by the incorporation of James-Martin compressibility factor. A factor of 0.5644 was used for the present experimental conditions (Ambrose, 1971). The specific retention volumes for the solutes show an inverse relationship with temperature, a common observation with other thermodynamic studies with GLC (Ambrose, 1971; Conder and Young, 1979; Purnell, 1962; King and List, 1990). In order to determine the temperature dependence, the data were plotted in Fig 5.1 for saturated fatty acid esters (FAE) and Fig 5.2 for unsaturated FAE. Specific retention volumes for all FAEs exponentially increased with decreasing temperature. It is also clear from the figures that V_s increased with the solute molecular weight and carbon number and decreased with an introduction of a double bond. The values of V_s for methyl esters of 18:1 and

18:2 were different only at lower temperature range (i.e. <220°C). Similar trend has been observed for their vapour pressures (Holman and Rahm, 1966).

Equation (5.4) suggests that a plot of log V_s versus log p^0 will be linear with a slope equal to unity, provided the stationary phase forms an athermal solution with the solute. This assumption was tested by plotting the literature values of log p^0 (Rose and Supina, 1961) as a function of log V_s for methyl ester of palmitic acid for the temperature range of 200 to 230°C (Fig 5.3). The resultant plot confirmed a linear relationship between the vapour pressure and the specific retention volume, however, the slope of the curve was not equal to unity. Therefore, FAEs do not form an athermal solution with the stationary phase and the heat of mixing is not zero. The slope of the curve is a fraction, $H_s/\Delta H_v$ (Majer et al., 1989). Accordingly, eq. (5.4) can be modified to give the following working equation (eq. 5.6), which allows calculations of constant 'A' i.e. $H_s/\Delta H_v$ and heat of mixing if enthalpy of vaporization is known. The constant 'B' includes the contribution due to non-ideality of solution and properties of the stationary phase.

$$\ln V_s = -A \ln p^0 + B \qquad (5.6)$$

Hoare and Purnell (1956) suggested the applicability of eq. (5.6) for calculations of boiling points for various solutes, e.g. hydrocarbons, alcohols and chlorinated hydrocar-

bons. Purnell (1962) noted that retention diagrams (log V_s versus log p^0) for esters show "family behaviour", i.e. the points fall on a common line. It follows therefore, that specific retention volumes can be used for the calculation of vapour pressures for unknown homologues or related solutes provided the relationship between V_s and p^0 for one of the homologues is known. Assuming that interactions between the liquid phase and FAEs are similar, vapour pressures for methyl and ethyl esters of various fatty acids were estimated. Tables 5.3 and 5.4 show the vapour pressures for selected saturated FAEs and unsaturated FAEs, respectively. Table 5.3 is based on calculations from eq. (5.7), obtained by regressing vapour pressure data (Rose and Supina, 1961) with corresponding V_s for methyl ester of palmitic acid.

$$\log p^0 = 5.160069 - 1.31263 \log V_s \quad (R^2 = 0.999) \qquad (5.7)$$

It was impossible to evaluate the goodness of estimations for all FAEs due to lack of experimental vapour pressure data at 200-230°C range. However, based on only a few experimental data, the estimations appear to be acceptable. For example, ethyl ester of palmitic acid was found to boil at 198.5°C at 13.5 mm Hg (Doss, 1952). According to the estimation from our study the boiling point for the same FAE is 200°C at 13.4 mm Hg.

Vapour pressures of unsaturated fatty acid esters given in Table 5.4 were estimated by eq. (5.8), which was based on

a linear relationship between experimental vapour pressures of 18:1 and 18:2 methyl esters with their specific retention volumes. The following regression equation represents a linear family plot for unsaturated FAEs

$$\log p^0 = 4.691046 - 1.17515 \log V_g \quad (R^2 = 0.995) \qquad (5.8)$$

It was assumed that for unsaturated FAEs, heat of mixing should be similar. Vapour pressure data for methyl esters of 18:1 and 18:2 were obtained from Boublik et al. (1973) for a range of temperatures between 200-230°C. Vapour pressures beyond the experimental range were obtained by extrapolation assuming a constant enthalpy of vaporization (ΔH_v). The assumption was valid as a plot of ln p^0 vs 1/T yielded a straight line for this range of temperatures.

Due to paucity of experimental data, it was difficult to test the accuracy of vapour pressure predictions for the FAEs studied. It was noted from estimations that vapour pressures of FAEs depended on carbon chain length, number of double bonds, and position of double bonds in the carbon chain. Vapour pressures of ω-6 FAEs were higher than those for corresponding ω-3 FAEs isomers at constant temperature. Thus, it may be possible to separate two structural isomers differing only in the position of first double bond both by judicious distillation and SFE. At constant temperature methyl esters had higher vapour pressures than the ethyl esters of the same fatty acids.

Linearity of a plot between log p^0 and corresponding log V_s (Figure 5.3) also suggest that this relationship can be used for both interpolation and extrapolation of limited vapour pressure data. Table 5.5 shows the comparison between the extrapolated vapour pressures and the literature values of methyl ester of palmitic acid (Boublik et al., 1973; Rose and Schrodt, 1963) at given temperatures. Specific retention volume was measured at both lower and upper ranges of temperature. The extrapolated values were lower than the literature vapour pressure and the deviation ranged from 4.8 to 9.7%. Percent deviation was higher at the temperature extremes (i.e. 180 and 250°C) suggesting that linearity is lost at these extremes. Also, at the higher temperature range some thermal degradation was likely. It may be recommended that extrapolations should only be performed at narrow range of temperatures.

Estimation of ΔH_v from GLC data

Specific retention volumes can be used for the calculation of ΔH_v of fatty acid esters from eq. (5.6), after substituting the integrated form of Clausius-Clapeyron equation for ln p^0 with an accompanying assumption that for a small range of temperature, ΔH_v remains constant. The modified equation will be:

$$2.303 \log V_s = \frac{H_s}{RT} + C \qquad (5.9)$$

A plot of log V_s versus 1/T (Figs 5.1 and 5.2) yielded a straight line of slope equal to H_s/R. Thus, the heat ofsolution can be readily calculated from such diagrams. It is often assumed that two non-polar compounds yield an athermal solution with ΔH_m being equal to zero. Since FAEs and dimethyl silicone polymers are essentially non-polar in nature, it is easy to assume that ΔH_m is zero. If such is the case H_s will be equal to ΔH_v, and Figures 5.1 and 5.2 can be used directly to calculate ΔH_v. In fact, this assumption is invalid for the present case. Table 5.6 shows calculated values for H_s, ΔH_v, and A for esters of 14:0, 16:0, 18:1, and 18:2 fatty acids. For these fatty acid esters, ΔH_v values were calculated from the experimental vapour pressure data (Boublik et al., 1973; Rose and Supina, 1961; Singleton, 1960) using Clausius-Clapeyron equation. Inequality of ΔH_v and H_s for these FAE indicate that FAE and dimethyl silicone polymer do not form an athermal solution. This corraborates the previous observation that the value of 'A', slope of ln V_s versus ln p^0, was different from unity. Fatty acid esters used in the study show positive deviation from Raoult's as 'A' were less than unity (Hoare and Purnell, 1956). The calculated ΔH_m for unsaturated FAEs were lower than those for saturated FAEs (Table 5.6). Therefore, unsaturated FAEs have much less interactions with the stationary phase than saturated FAEs. Since ΔH_m is not constant and varied among the FAEs, it was not possible to calculate ΔH_v for 20:5 and 22:6 esters.

However, the method can be employed for calculation of ΔH_v for esters whose vapour pressures and specific retention volumes are known, without recourse to Clausius-Clapeyron equation, or when calorimetric ΔH_m values are known.

Conclusion

Comparison of experimental normal boiling points and estimations from group contribution method of Meissner indicated that the method underestimates normal boiling points of fatty acids and their esters. This method should be avoided for saturated fatty acids of lower carbon numbers. However, the estimations for unsaturated fatty acids are acceptable within desired accuracy.

A non-destructive and simple method using GLC was described for determination of vapour pressures of esters of fatty acids of biological importance. Vapour pressures of methyl esters of ω-3 fatty acids, eicosapentaenoic acid (20:5) and docosahexanenoic acid (DHA, 22:6) and ethyl ester of DHA were estimated from the GLC retention data. It was also shown that, provided log V_s and log p^0 are linearly related (constant H_s), experimental V_s could be used for extrapolation of vapour pressures within a narrow temperature range. The method is capable of predicting differences in vapour pressures for structural isomers of FAEs, e.g. γ-linolenic acid esters have higher vapour pressures than its α-isomer. In this respect, the GLC method is superior to group contribution method, which

does not take into account the contribution of these differences to boiling points.

Gas liquid chromatography can not be used for the determination of ΔH_v directly from retention data, particularly when dimethyl silicone is a stationary liquid phase, as ΔH_m is not negligible. However, it is possible to estimate ΔH_m for regular solutions via infinite dilution activity coefficient (γ^s) and eq. (5.4), provided an exact molecular weight of the liquid phase is known. ΔH_m can also be independently measured by calorimetry. From known ΔH_m, it is indeed possible to calculate ΔH_v with the proposed method.

Table 5.1. Comparison of experimental normal boiling points (K) of fatty acids and their esters with estimates from Meissner equation.

Fatty acid or ester	Literature[1]	Estimate from equation (5.5)	% Absolute Error*
14:0	599.3	565.6	5.6
16:0	624.6	594.2	4.9
18:0	649.2	621.6	4.2
18:1	638.0	626.6	1.8
18:2	638.3	631.7	1.0
14:0 ME	568.9[2]	552.9	2.8

* % error = 100 x (calculated-experimental)/experimental

ME methyl ester

1 Data from Singleton (1960)

2 Data from Perry and Chilton (1973)

Table 5.2. Specific retention volumes (mL/g) for fatty acid esters at selected temperatures.

Fatty acid ester	Temperature (°C)			
	200	210	220	230
Methyl ester				
16:0	935.3	691.2	529.1	410.6
18:1	1692.8	1211.4	889.3	662.7
18:2	1641.3	1179.1	867.3	666.8
20:5 ω-3	2862.8	1985.6	1460.2	1066.2
22:6 ω-3	5262.0	3548.6	2496.8	1787.1
Ethyl ester				
14:0	599.3	457.6	356.7	285.3
16:0	1178.8	863.3	643.9	494.4
18:0	2358.8	1650.9	1185.4	874.5
22:6 ω-3	6500.1	4346.9	3003.4	2136.2

Table 5.3. Vapour pressures of saturated fatty acids esters calculated from equation (5.7).

Fatty acid ester	Temperature (°C)	Vapour Pressure (mm Hg)
14:0 EE	200	32.7
	210	46.5
	220	64.5
	230	86.5
16:0 EE	200	13.4
	210	20.2
	220	29.7
	230	42.0
18:0 EE	200	5.4
	210	8.6
	220	13.3
	230	19.9
19:0 ME	200	4.6

ME methyl ester

EE ethyl ester

Table 5.4. Vapour pressures of unsaturated fatty acid esters calculated from the measured specific retention volumes

Fatty acid ester	Temperature (°C)	Vapour Pressure (mm Hg)
20:5 ω-3, ME	200	4.3
	210	6.5
	220	9.4
	230	13.6
22:6 ω-3, ME	200	2.1
	210	3.3
	220	5.0
	230	7.4
22:6 ω-3, EE	200	1.6
	210	2.6
	220	4.0
	230	6.0
18:1 EE	200	6.8
18:2 EE	200	5.9
18:3 ω-3, ME	200	7.9
18:3 ω-6, ME	200	8.6
18:3 ω-3, EE	200	4.3
18:3 ω-6, EE	200	6.3
20:2 ME	200	4.6
20:3 ME	200	3.6
20:4 ME	200	3.5

MM methyl ester

EE ethyl ester

Table 5.5. Experimental and extrapolated vapour pressures of methyl ester of palmitic acid obtained from GLC specific retention volume data.

Temperature (K)	V_s (mL/g)	Vapour Pressure (mm Hg)		
		A	B	Abs.Err. (%)
453.15	1854.0	7.4	8.2 [a]	9.7
463.15	1304.9	11.8	12.4 [a]	4.8
513.15	327.4	72.2	77.9 [b]	7.3
523.15	263.7	95.9	104.6 [b]	8.3

A Vapour pressures calculated by extrapolation.

B Literature data.

[a] Calculated from the Antoinne constants given by Boublik et al. (1973).

[b] Calculated from the Antoinne constants given by Rose and Schrodt (1963).

Table 5.6. Enthalpy of vaporization (ΔH_v), enthalpy of solution (H_s), constant 'A' for fatty acid esters on dimethyl silicone liquid phase at 200 to 230°C.

Fatty acid ester	H_s [a] kJ/mol	ΔH_v [b] kJ/mol	ΔH_m [c] kJ/mol	A[d]
14:0 EE	49.0	71.4	22.4	0.686
16:0 ME	54.2	71.3	17.1	0.761
16:0 EE	57.4	74.2	16.8	0.778
18:1 ME	61.8	71.8	10.0	0.861
18:2 ME	59.6	71.3	11.7	0.836
20:5 ME	64.8	-	-	-
22:6 ME	71.1	-	-	-
22:6 EE	73.4	-	-	-

[a] Calculated from eq. (5.9).

[b] Calculated from literature vapour pressure data (Boublik et al., 1973; Rose and Supina, 1961)

[c] $\Delta H_m = \Delta H_v - H_s$

[d] Calculated from eq. (5.6).

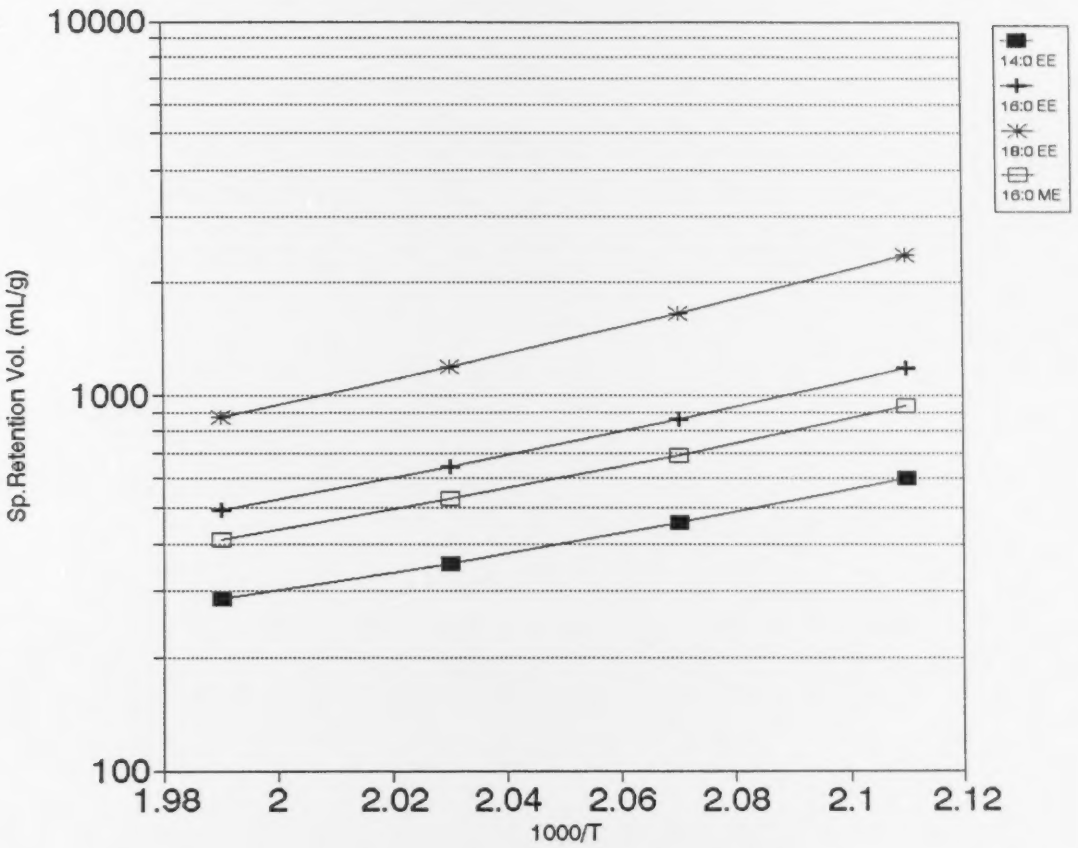

Figure 5.1. Effect of temperature on the specific retention volumes of saturated fatty acid esters.

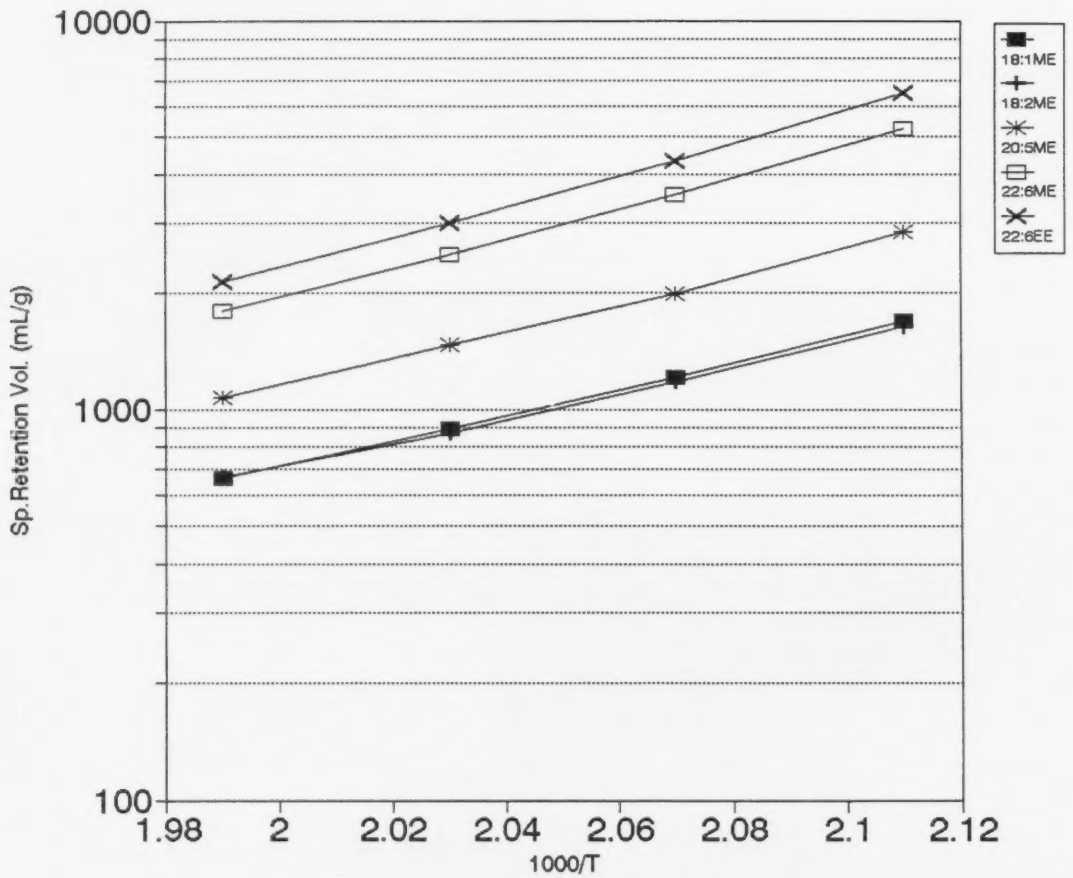

Figure 5.2. Effect of temperature on the specific retention volumes of unsaturated fatty acid esters.

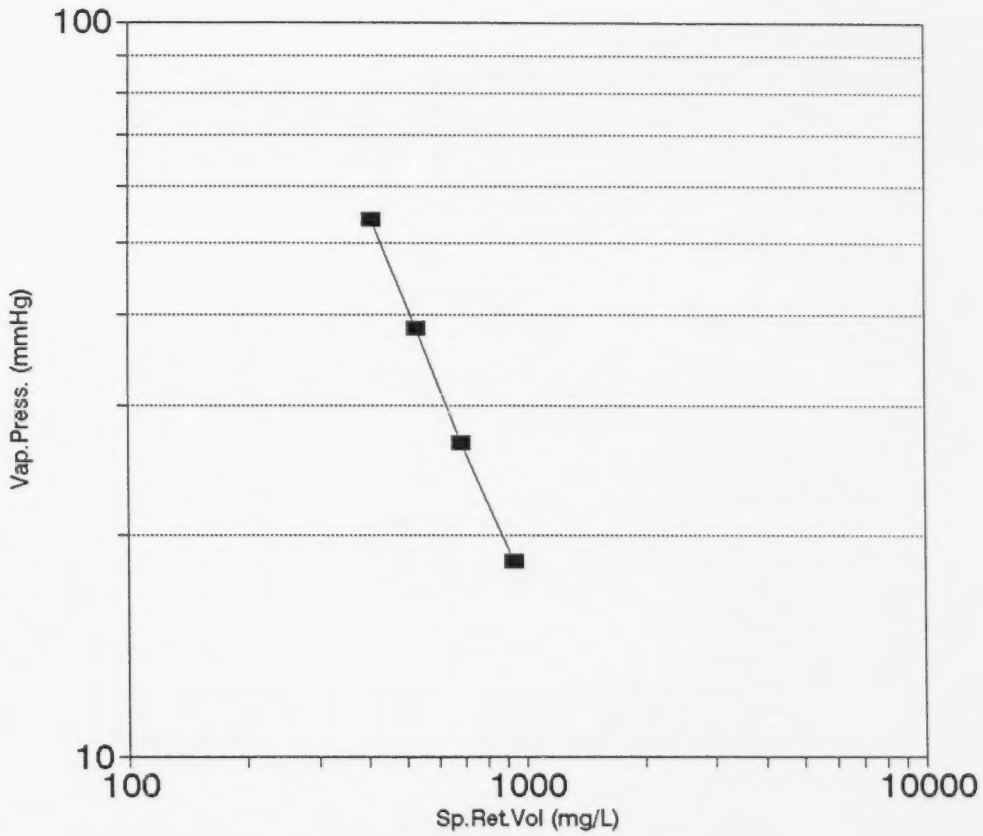

Figure 5.3 Vapour pressure-specific retention volume plot for palmitic acid methyl ester.

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6. MODELLING BINARY PHASE BEHAVIOUR OF SUPERCRITICAL CARBON DIOXIDE AND FATTY ACID ESTERS[1]

Introduction

There has been increased interest in separation and fractionation of fatty acids due to their role in human health and diseases. Omega-3 (ω-3) fatty acids have been noted for their beneficial role in reducing the risk of various heart diseases (Kinsella, 1986). Due to their complex chemical nature, conventional separation methods, e.g. distillation, are undesirable and inefficient. Supercritical carbon dioxide (SC CO_2) has been investigated for extraction and fractionation of these fatty acids and their esters (Eisenbach, 1984; Bharat et al., 1989; Liong et al., 1992; Krukonis, 1988a and 1988b; Nilsson et al., 1988, 1989a, 1989b and 1990; Rizvi et al., 1988). Although, these studies have proven the technical feasibility of using SC CO_2 for extraction and fractionation of fatty acids at laboratory and pilot plant scales, the process is yet to be commercialized.

Data on phase behaviour of fatty acid esters in SC CO_2 are a prerequisite to the design, development and optimization of extraction processes on commercial scales. Since direct experimental generation of equilibrium solubility data is

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[1] A version of this study has been submitted for publication in J. Supercritical Fluids (Mishra et al., 1992).

costly, difficult and time consuming, it is often desirable to predict the data with a suitable model. Development of thermodynamic models to predict the solubility of solutes in supercritical fluids (SCF) is hampered by a lack of understanding of intermolecular interactions between the SCF and solute. The asymmetric nature of the mixture and a lack of physical properties in the case of biomaterials add to the severity of the problem.

In nature, fatty acids occur as esters of glycerol. It is advantageous to transesterify these glyceryl esters to methyl or ethyl esters for fractionation. Solubilities of methyl and ethyl esters are higher than those of triglycerides due to about a four fold increase in vapour pressure and the reduction in molecular weight (Rizvi et al., 1988). Therefore, methyl and ethyl esters of fatty acids have been found to be better feedstocks for fractionation by SCFs (Rizvi et al., 1988a). Furthermore, ethyl esters are preferred over methyl esters as a concentrated source of fatty acids intended for human consumption since methyl esters produce methanol as a metabolic product, which is toxic.

The objective of the present study is to develop a thermodynamic model based on regular solution theory to predict the solubility of two ω-3 fatty acid ethyl esters, i.e. eicosapentaenoic (EPA, 20:5 ω-3) and docosahexaenoic acids (DHA, 22:6 ω-3) in SC CO_2.

Theory

The solubility of any solute in a SCF can be determined by equating the fugacities for all components (i) in every phase in equilibrium

$$f_i^I = f_i^{II} = f_i^{III} \ldots \tag{6.1}$$

Calculation of fugacities is accomplished by constitutive equations based on the components of a mixture and phases in equilibrium. Molecular complexity of biomaterials and their mixtures make this task difficult. The fluid phase fugacity (f_2^F) can be evaluated from the volumetric properties

$$f_2^F = y_2 \, \phi_2^F \, P \tag{6.2}$$

Alternatively, fugacity can also be calculated using solution thermodynamic approach from

$$f_2^F = y_2 \, \gamma_2^F \, f_2^0 \tag{6.3}$$

The activity coefficient (γ) in eq. (6.3) and the fugacity coefficient (ϕ) in eq. (6.2) account for non-idealities relative to the standard state; ideal solution and ideal gas behaviour, respectively. The value of ϕ encountered in SFE is much less than 1, leading to a large solubility enhancement in SCF in comparison to ideal gas consideration only (Mackay and Paulaitis, 1979). Fugacity coefficient is related to volumetric properties of a mixture (Prausnitz et al., 1986) as

$$RT \ln\varphi_i = \int_v^\infty [(\frac{\partial P}{\partial n_i})_{T,V,n_j} - \frac{RT}{V}] dV - RT \ln Z \qquad (6.4)$$

The use of eq. (6.2) necessitates availability of volumetric data on mixtures, which are often absent. However, fugacity coefficient can be calculated from the theory of corresponding states, a suitable equation of state (EOS), or from generalized partition functions (Prausnitz et al., 1986). Various EOSs have been proposed for this purpose. At moderate pressures, the virial equation can be used, however, unavailability of higher order virial coefficients prevent their use for quantitative prediction of high pressure phase behaviour. One of the most popular approaches is to use cubic EOSs for calculation of φ for vapour and liquid phases with the assumption that EOS is valid for both phases. Successful use has been made of modified versions of van der Waal's equation, e.g. Redlich-Kwong, Soave's modification of Redlich-Kwong and Peng-Robinson equation. Mathematical expressions of fugacity coefficients from various cubic EOS are given by Reid et al. (1987). Adoption of an EOS for a mixture requires mixing rules for calculations of EOS parameters. The accuracy of prediction of phase equilibria of mixtures from EOS is therefore, intimately related to the accuracy with which these parameters are obtained using mixing rules. Mixing rules contain binary interaction parameters which need to be calculated from the experimental phase data. Besides being

empirical in nature, cubic EOSs are therefore, a scheme to collate experimental data.

Scatchard-Hildebrand regular solution theory (RST) has been widely employed for qualitative prediction and correlation of phase equilibrium data involving non polar-liquid solvents. The use of this theory in SFE and supercritical chromatography (SFC) was demonstrated by several researchers (Giddings et al., 1968; Stahl et al., 1978; Eissler and Friedrich, 1988; King, 1983 and 1984). One of the advantages of RST is its ability to correlate a solute's molecular structure to its solubility in a SCF, thereby avoiding the need for costly and difficult experimentation involved in feasibility studies. It finds application not only in screening solvents (King, 1980), but also can be used for the selection of optimum extraction conditions. The optimum conditions of extraction pressure and temperature will be those corresponding to equality of solubility parameters (δ) of solvent and solute (King, 1983).

Solubility parameter is defined as the square root of internal pressure or cohesive energy density (Fedors, 1974):

$$\delta = \sqrt{-\frac{E}{V}} = \sqrt{\frac{(\Delta H_v - RT)}{V}} \qquad (6.5)$$

Cohesive energy (E) is related to the enthalpy of vaporization (ΔH_v) at temperatures remote from the critical temperature[2]. Solubility parameter can be used to calculate solubility of a solute in a liquid solvent via activity coefficients (Prausnitz et al., 1986). The concept of δ has been applied to phase equilibria under supercritical conditions by treating the SCF as an expanded liquid and assuming the nonideality to be only due to differences in van der Wall's forces among the species present. Following this model, enhancement of solubility of heavy hydrocarbons and acids have been correlated by several researchers (Mackay and Paulaitis, 1979; Ziger and Eckert, 1983; Kramer and Thodos, 1988a, 1988b and 1989; Gurdial and Foster, 1991). Theoretical aspects of solubility parameter in relation to phase behaviour at high pressures have been reviewed by Rizvi et al. (1988b).

Thermodynamic Model

The solubility of fatty acid esters (component 2) in SC CO_2 (component 1) can be determined by writing eq. (6.3) for both phases and substituting into eq. (6.1)

$$y_2 \gamma_2^F f_2^{0F} = x_2 \gamma_2^L f_2^{0L} \qquad (6.6)$$

Since, SC CO_2 is assumed to be an expanded liquid the standard state fugacities (f^{0F} and f^{0L}) in both phases should

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[2] ΔH_v of a component at its critical points is equal to zero.

be equal and cancel out. Furthermore, eq. (6.6) can be rearranged to yield the equilibrium distribution coefficient (K_2) of the fatty acid ester in SC CO_2

$$K_2 = \frac{y_2}{x_2} = \frac{\gamma_2^L}{\gamma_2^F} \qquad (6.7)$$

Distribution coefficient can be predicted theoretically provided the fluid and liquid phase activity coefficients can be obtained from a suitable model. Regular solution theory has been used successfully for the estimation of activity coefficients in liquid phase for non polar components like hydrocarbons (Chao and Seader, 1961). Czubryt et al. (1970) have used RST for qualitative prediction of solubility of various solutes including stearic acid. Kramer and Thodos (1988a, 1988b and 1989) estimated activity coefficients of fatty alcohols and their fatty acids at infinite dilution in SC CO_2. If a mixture of a fatty acid ester and SC CO_2 may be assumed to form a regular solution, then γ can be calculated as suggested by Hildebrand and Scott (1962) from the pure component properties i.e. solubility parameters

$$RT \ln\gamma_2 = V_2\phi_1^2(\delta_1-\delta_2)^2 \qquad (6.8)$$

The volume fraction Φ_1 is calculated by assuming additivity of volumes for the binary mixture under consideration. In case of a liquid phase infinitely dilute in component 2 with respect to component 1, Φ_1 is equal to unity.

The expression for activity coefficient for infinite dilution (γ^{∞}) simplifies to

$$RT \ln\gamma_2^{\infty} = V_2(\delta_1-\delta_2)^2 \qquad (6.9)$$

Equation (6.9) does not require any experimental data as long as the solution is infinitely dilute as assumed by Kramer and Thodos (1988a, 1988b, and 1989). For the present investigation, the concentration of FAE in SC phase (light phase) was considered to be infinitely dilute. However, due to considerable solubility of SCF in the liquid ester phase, the activity coefficient of solute in the FAE (heavy) phase required incorporation of a volume fraction term. After substituting eq. (6.8) for γ_2^L and eq. (6.9) for γ_2^F in eq. (6.7) the resulting expression for K values of FAE (component 2) will be the following equation

$$\ln K_2 = \frac{V_2}{RT}(\delta_1-\delta_2)^2(\phi_1^2-1) \qquad (6.10)$$

Parameter estimation

Use of this model requires a knowledge of solubility parameters for SC CO_2 and FAEs, molar liquid volume of FAE and composition data of the FAE liquid phase. The solubility parameter of SC CO_2 was calculated as suggested by Giddings et al. (1968)

$$\delta_1 = 1.25\sqrt{P_c}\frac{\rho_{R_1}}{\rho_{R_L}} \qquad (6.11)$$

The reduced density of SC CO_2 was from the density data obtained from Bruno and Svoronos (1989).

Properties of EPA and DHA ethyl esters were estimated by a group contribution method (Fedors, 1974). A computer program was written for the calculation of both molar volumes and solubility parameters of EPA and DHA ethyl esters at 25°C. Fedors's method underpredicted the molar volumes of fatty acid esters. A comparison of experimental and predicted molar volumes is given in Table 6.1. Assuming the errors are evenly distributed the experimental values of molar volumes (Singleton, 1960) were regressed with the predicted ones to obtain the following equation

$$V_{corr} = 104.0352+0.75827V_{gc} \quad (R^2 = 0.99) \qquad (6.12)$$

Eq. (6.12) was used for correction of molar volumes of EPA and DHA ethyl esters. Predictions from Fedors's group contribution method showed an average absolute error of 8% in comparison to 0.02% by eq. (6.12). Corrected values were used for calculation of solubility parameters. Molar volumes and solubility parameters of EPA and DHA ethyl esters were 325.75 cm^3/mol and 9.0 $(cal/cm^3)^{0.5}$; and 335.23 cm^3/mol and 9.18 $(cal/cm^3)^{0.5}$, respectively. Solubility parameters for the FAEs can also be calculated using eq. (6.5) from ΔH_v values obtained from gas chromatographic data (Chapter 5). Since ΔH_vs calculated from the gas chromatographic retention data were for a temperature range well above the range under

consideration for this investigation, it was not possible to use eq. (6.5) for determination of solubility parameters of FAEs. Therefore, group contribution technique was preferred over gas chromatographic determination of ΔH_v as the latter method required extrapolation of vapour pressure-temperature data to 25°C, a region where the curve between vapour pressure and temperature becomes non-linear.

Experimental composition data for FAE liquid phase were adapted from Bharat et al. (1989) for calculation of Φ_1 and for comparison of predictions obtained by eq. (6.10).

Results and Discussion

The concept of regular solution theory has been extended to describe solvation capacity of SCFs (Allada, 1984; King, 1984). Allada (1984) prepared generalized solubility parameter charts and showed that this approach could be used for qualitative prediction of the mechanism of solubility in SCFs. It was suggested that δ rather than pressure and temperature was the key determinant of solubility of fuel-oil residues in SCFs (Allada, 1984). This approach was adopted to study solubility of EPA and DHA ethyl esters in the SC CO_2 at different pressures and temperatures of 40, 50, and 60°C. Figures 6.1 and 6.2 show the solubility of ethyl esters of EPA and DHA, respectively, as a function of δ of SC CO_2 (δ_1). For both systems, δ_1 alone failed to describe the temperature effects on solubility, which suggest that intermolecular

interactions between the FAEs and SC CO_2 were sensitive to changes in temperatures. Eissler and Friedrich (1988) made similar observations with oil-SC CO_2 system. However, each solubility isotherm can be described by a linear relationship of the following type

$$\log y_2 = A + B\delta_1 \qquad (6.13)$$

The coefficients A and B in eq. (6.13) are functions of temperature. Both coefficients were determined by regressing log y_2 (Bharat et al., 1989) as a function of δ_1 at 40, 50, 60°C and are given in Table 6.2. In all cases the correlation coefficient (R^2) was greater than 0.99. It is apparant from Table 6.2 that the slopes of the solubility isotherms (B) were a weaker function of temperature than the intercepts (A).

Theoretically, equality of δs of solute and solvent correspond to maximum solubility, hence it is possible to determine optimum extraction conditions for maximum solubility. Fujishiro and Hildebrand (1962) showed that compositional data can be used for the calculation of solubility parameter difference between the solute and solvent. Vapour phase mole fraction of EPA EE and DHA EE were plotted as a function of the solubility parameter difference between SC CO_2 and esters on semilogarithmic coordinates in Figs. 6.3 and 6.4, respectively. These solubility isotherms clustered around a single line indicating a possibility that the vapour phase composition was dependent on the solubility parameter differ-

ence for all temperatures. The trend is less obvious in the case of DHA EE (Fig. 6.4), perhaps due to a much larger scatter in the experimental data points. This behaviour makes possible the calculation of the solubility of these fatty acid esters from the solubility parameter difference at different temperature-pressure combinations, provided experimental data are available for only one isotherm. This is of considerable significance, since conducting experiments with these FAEs is not only costly, but also difficult.

Despite its usefulness, the above approach is empirical as it relies heavily on the properties of SC CO_2 and disregards the FAE liquid phase completely. On the contrary, the liquid phase composition is important in determining the solubility of FAE into fluid phase by eq. (6.10). Experimental data of Bharat et al. (1989) show that an increase in solubility of these FAEs measured at high pressures is in correspondence with an increase in the solubility of CO_2 in the ester liquid phase. Brunner and Peter (1982) made a similar observation for triglyceride, fatty acids and SC CO_2 systems. Solubility of CO_2 in the liquid phase was accounted for in terms of the equilibrium distribution coefficient (K) of FAEs. In this study, regular solution theory was extended to predict experimental K values of EPA and DHA ethyl esters by eq. (6.10). The results of this model were compared with the experimental K values, calculated from the compositional data of Bharat et al. (1989) for the studied FAEs (Figs. 6.5

and 6.6). Within the range of conditions under investigation, the model correctly predicted the observed increase in solubility at higher pressures and lower temperatures for EPA and DHA EEs. In general, the quantitative prediction of experimental K values was reasonable. Even though at conditions close to the critical point of SC CO_2, the model performed poorly, the agreement between experimental and predicted values was best at 60°C. Errors in prediction may be in part due to the fact that in the derivation of eq. (6.10) the volume change upon mixing in the liquid phase was neglected completely. It is also known that the partial molar volume of these FAEs become increasingly negative at high pressures and low temperatures indicating solvent clustering (Liong et al., 1991), a phenomenon unexplained by RST.

Conclusion

Vapour phase solubility of ethyl esters of EPA and DHA in SC CO_2 is exponentially related to the solubility parameter of SC CO_2 at constant temperature. Solubility parameter of the solvent alone could not explain the effect of temperature on the solubility.

Regular solution theory was extended further to model the binary vapour liquid equilibria of SC CO_2 and ethyl esters of EPA and DHA assuming the light phase to be an expanded liquid. The model correctly predicted a decreasing trend in logarithm of the equilibrium distribution coefficients of EPA and DHA

ethyl esters in supercritical phase as a function of extraction temperature. Except at conditions close to critical point of CO_2, the estimates were in qualitative agreement with the experimental data of Bharat et al. (1989).

Table 6.1. Comparison of experimental and predicted molar volumes (cm^3/mol) of selected fatty acid esters at 25°C.

Fatty acid ester	V (cm^3/mol)		
	Experimental	Predicted (Fedor's)	Predicted Eq.(6.12)
18:1 ME	340.4	322.9	348.9
18:1 ME	359.0	339.0	361.0
18:2 ME	332.3	303.2	333.9
18:3 ME	326.5	283.5	319.0
22:1 ME	402.3	387.3	397.7

ME Methyl ester

EE Ethyl ester

a From Singleton (1960)

Table 6.2. Parameters for calculation of solubility of EPA and DHA esters in SC CO_2 by eq. (6.13)[1].

Temperature (°C)	A		B	
	EPA EE	DHA EE	EPA EE	DHA EE
40	-6.2945	-6.1993	0.66811	0.61232
50	-5.8446	-6.3032	0.60878	0.6457
60	-5.7726	-5.9434	0.61742	0.60999

EPA EE eicosapentaenoic acid ethyl ester

DHA EE docosahexaenoic acid ethyl ester

[1] range of δ_1 = 3-7 $(cal/cm^3)^{1/2}$

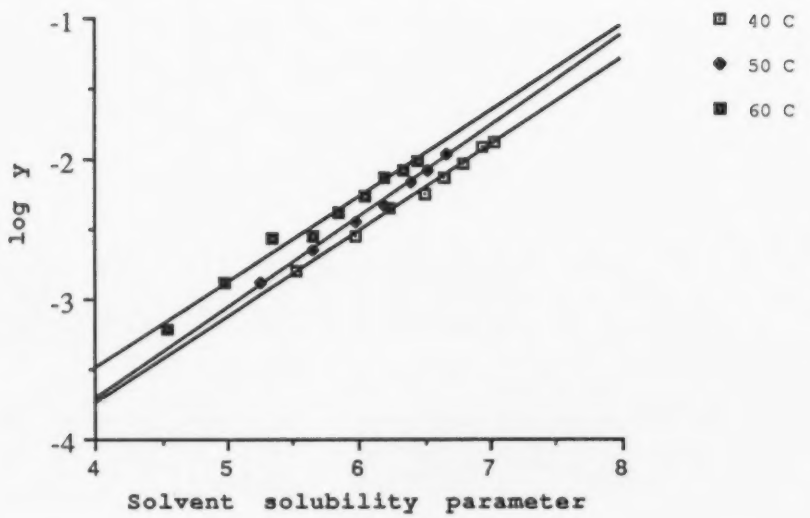

Figure 6.1. Effect of solubility parameter of SC CO_2 on the solubility of ethyl ester of EPA at 40, 50 and 60°C (solubility data from Bharat et al. (1989)).

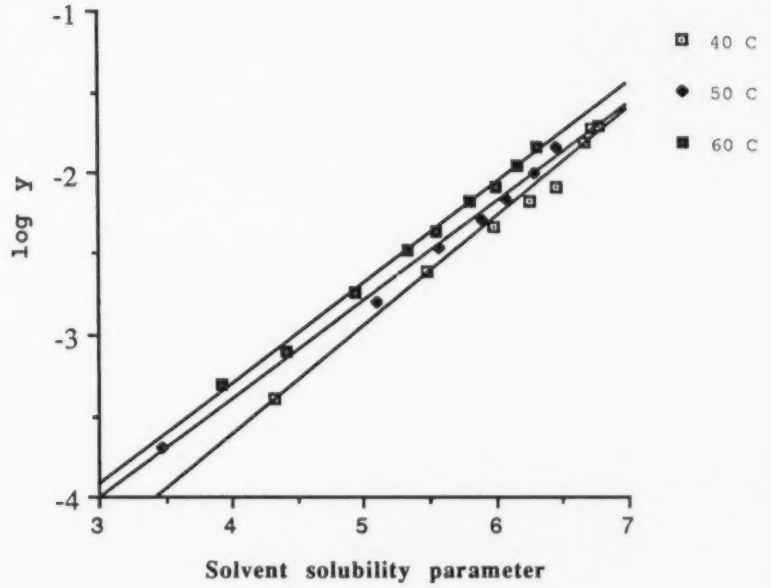

Figure 6.2. Effect of solubility parameter of SC CO_2 on the solubility of ethyl ester of DHA at 40, 50 and 60°C (solubility data from Bharat et al. (1989)).

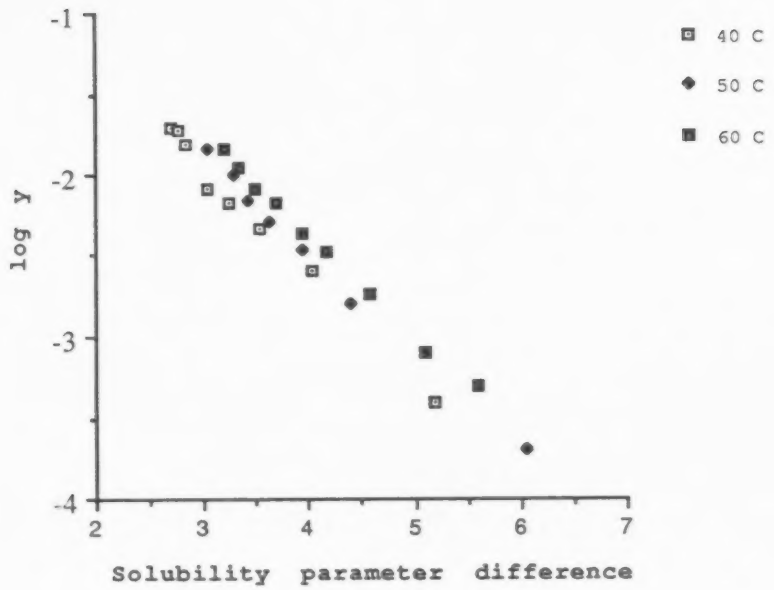

Figure 6.3. Effect of solubility parameter difference between SC CO_2 and ethyl ester of EPA at 40, 50 and 60°C (data from Bharat et al. (1989)).

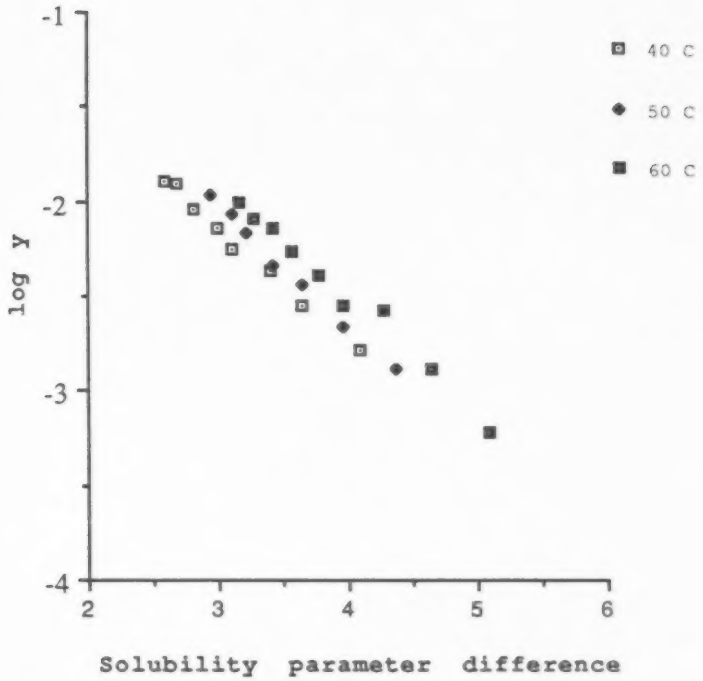

Figure 6.4. Effect of solubility parameter difference between SC CO_2 and ethyl ester of DHA at 40, 50 and 60°C (data from Bharat et al. (1989)).

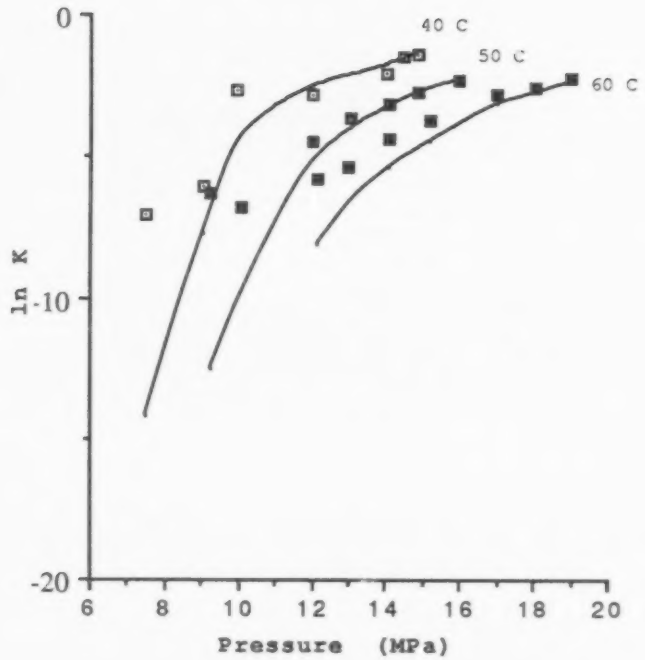

Figure 6.5. Experimental (□) and predicted (——) K values at 40, 50 and 60°C for ethyl ester of EPA (data from Bharat et al. (1989)).

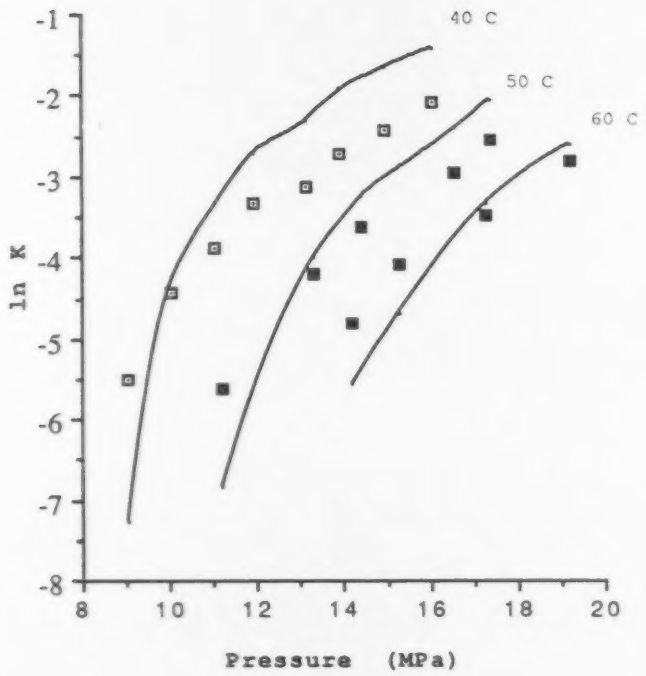

Figure 6.6. Experimental (□) and predicted (——) K values at 40, 50 and 60°C for ethyl ester of DHA (data from Bharat et al. (1989)).

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7. CONCLUSIONS AND RECOMMENDATIONS

There has been ample evidence in literature about the beneficial effects of consuming ω-3 polyunsaturated fatty acids (PUFA) particularly, eicosapentaenoic (EPA 20:5, ω-3) and docosahexaenoic acids (DHA 22:6, ω-3) (Kinsella, 1987). However, most clinical studies are based on dose response analysis, which include combined effects of mixtures of EPA, DHA and other PUFAs e.g. arachidonic acid (20:4, ω-6). Hence, it has been difficult to separate the biological effects of EPA or DHA in order to understand the underlined mechanism of action of ω-3 PUFA. Extensive studies are being conducted in this area and fatty acids concentrated in EPA or DHA are in demand by clinicians (Dyerberg, 1986).

The main objective in our study was to use dulse (Palmaria palmata) as a raw material for production of EPA rich oil concentrate. Compositional analysis of wild and cultured mutant of dulse has shown significant differences in protein, ash and carbohydrate contents, however lipid content and fatty acid profile were identical for the two strains. By virtue of its high protein (27%) and mineral (27%) contents, cultured strain can be regarded as an ideal source of these nutrients rather than ω-3 PUFA. Eicosapentaenoic acid content of the analyzed strains was low (45%) as compared to previously reported value of 71% (Ackman, 1981). The cellular composition of the mutant strain was affected by cultural conditions. The optimum cultural conditions identified for maximum

accumulation of EPA were as follows: temperature 11°C; harvest time 20 days; and nitrogen supplemention. Other factors that may influence lipid and EPA contents are salt content of media, light intensity and vitamin supplementation (Yonmanitchi et al., 1991). Further study is needed to ascertain the role of these factors. Based on the results reported in chapter 2, it appears that lipid content and fatty acid composition is controlled more by genetic than environmental factors. Genetic manipulation of the algae is, therefore, needed to increase its lipid content.

The freeze dried powder (FDP) of dulse prepared for lipid extraction was characterized as low bulk density and cohesive with considerable anisotrophy. Low bulk density not only necessitated higher extraction vessel volume, but also the problems of particle entrainment and compaction resulted in poor lipid recovery by supercritical fluid extraction (SFE). The powder was found to be extremely hygroscopic. Extreme care must be observed during storage and handling as the powder may cake upon exposure to humid atmospheres. Adsorption isotherm for FDP was described by BET model at water activity (a_w) less than 0.5, and by Harkin-Jura-Smith model at a_w above 0.5. The BET monomolecular value for this material was 4.8 kg water/100 kg of dry powder. It is recommended that FDP be stored near this moisture content to ensure lipid stability.

It was technically possible to extract lipids from FDP using SC CO_2. The extract obtained by lean SC CO_2 did not contain chlorophyll and polar lipids. Lipid solubilities were higher at low pressures and did not increase by increasing extraction pressure. Similar observations have been made earlier (Schmitt and Reid, 1986; Polak et al., 1989; Vijayan and Buckley, 1991). Influence of caking and compaction during extraction on poor recovery of lipids at higher range of pressure can not be overruled. The study also showed that some of the lipid in dulse may be bound to the solid matrix and thus was inaccessible to SC CO_2 for extraction. It was not possible to explore this anomalous behaviour further due to material and time constraints and, therefore, should be a subject for future study.

Dulse was found unsuitable as a raw material for extraction of ω-3 fatty acids, despite its high concentration of EPA, due to its low lipid content. It is not recommended as a source of these fatty acids unless more work is devoted to increase the total lipid content, e.g. through genetic engineering.

ω-3 fatty acids are absorbed in the human body preferentially as triglycerides (El-Boustani et al., 1987). Therefore, we had planned our initial studies to extract lipids rich in EPA so that no further purification steps would be necessary before their consumption. Due to problems encountered as described, it appears more feasible to adopt the following

processing sequence: extraction of lipids by solvents, transesterification, fractionation and concentration of fatty acid esters using SC CO_2 and synthesis of triglycerides from fatty acid concentrate. The fatty acid profile of dulse is particularly suited for concentration of EPA for two reasons:

1. The concentration of other fatty acids with a carbon number higher than 20 is negligible, and
2. The concentration of EPA in C20 fraction is extremely high (>90%).

Keeping this in perspective, the information about the physical properties of fatty acid esters relevant for SFE was generated for some ω-3 PUFA. Vapour pressures of fatty acid esters (FAE) are an important determinant of solubility in SC CO_2 (McHugh and Krukonis, 1986) and are needed for comparison of SFE with distillation as a method of concentration. In general, higher volatility translates into higher solubility. In this study (chapter 5), the group contribution method of Meissner (Meissner, 1949) was evaluated for its capability to predict the normal boiling points of fatty acids. Comparison of experimental and estimated boiling points indicated that normal boiling points for polyunsaturated fatty acids could be estimated with acceptable engineering accuracy. Also, a non destructive and simple method was described for determination of vapour pressure of fatty acid esters from gas chromatographic retention volume data. A correlation was presented for estimation of vapour pressures of saturated and unsatu-

rated fatty acids. For a range of temperature of 200 to 230°C, vapour pressures were estimated for PUFA including EPA and DHA esters for the first time. The method was also found to be capable of predicting the difference in vapour pressures for structural isomers. It was found that the higher the ω number the higher will be the vapour pressure and consequently higher will be the solubility in SC CO_2 (Nilsson et al., 1991). Due to significant enthalpy of mixing between the ester and poly methyl silicone stationary phase and paucity of physical property data of the stationary phase, it was not possible to calculate enthalpy of vaporization of FAEs directly from retention data. The vapour pressure data generated can be used for simulation of distillation pattern of fatty acid esters.

Assuming SC CO_2 as an expanded liquid, the binary phase behaviour of ethyl esters of EPA and DHA in SC CO_2 were modelled using regular solution theory. The vapour phase solubilities of the esters in SC CO_2 (Bharat et al., 1989) were successfully correlated with solvent solubility parameter. Correlation for each isotherm was presented. The advantage of this approach is its reliance only on the properties of solvent, which have been worked out thoroughly. In order to include the effects of solubility of CO_2 in the ester phase, and make the model more rigorous, the regular solution theory was extended further for calculation of equilibrium distribution coefficients for EPA and DHA ethyl

esters in SC CO_2. The model is particularly attractive as it requires minimum data on properties. Costly and often difficult experimentation can be avoided as the model can be used as a first step in designing feasibility studies. The model is also capable of predicting the solubility trend observed at high pressure and temperature conditions for both EPA and DHA ethyl esters. The predictability of the model can be improved further by including a mixture parameter. However by such a treatment, the simplicity of the model is lost as mixture properties are usually not available.

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Appendix I

Table I.1 Mesh number and corresponding diameter of opening of the Canadian Standard Sieves used for sieve analysis.

Mesh number	Diameter of the opening (μ)
35	500
40	425
60	250
80	180
100	150
120	125

Table I.2 Saturated salt solutions used for achieving a constant water activity at 23°C (Rockland, 1960).

Name	Formula	Water activity
Phosphorous pentoxide*	P_2O_5	0.00
Lithium chloride	$LiCl_2.H_2O$	0.11
Potassium acetate	CH_3COOK	0.23
Calcium chloride	$CaCl_2$	0.32
Magnesium nitrate	$Mg(NO_3)_2$	0.52
Cupric chloride	$CuCl_2$	0.67
Potassium chloride	KCl	0.86
Water	H_2O	1.00

* used as such.